PHOSPHORYLATION AND SUMOYLATION HELP MAINTAIN THE FUNCTIONS OF ENDOTHELIAL CELL-EXPRESSED LEUKOCYTE-SPECIFIC PROTEIN 1 (LSP1)

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ABSTRACT

Post-translational modifications are decisive in providing functional maturation and diversity of many proteins. Leukocyte-specific protein 1 (LSP1) plays an important role in the regulation of endothelial permeability, leukocyte chemotaxis and transendothelial migration during inflammation. Unlike leukocyte-expressed LSP1 which is predominantly a cytosolic protein, endothelial cell-expressed LSP1 is mainly localized in the nucleus. It's translocation to extranuclear compartments is promoted by tumor necrosis factor-α (TNF-α). There is a discrepancy between the predicted molecular weight of LSP1 (37 kDa) and its actual appearance in SDS-PAGE (> 50 kDa). All of these discrepancies indicate possible post-translational modifications. In spite of its important role in acute inflammation and these apparent indications, very little is known about post-translational modifications of endothelial cell-expressed LSP1. To address this, we investigated whether endothelial cell-expressed LSP1 activation is linked to its phosphorylation by p38 mitogen activated protein kinase (p38 MAPK) and protein kinase C (PKC) upon chemokine treatment of endothelial cells and whether it requires additional signaling such as leukocyte-endothelial cell interaction. The present study also addresses how post-translational modifications help maintain the functions of endothelial cell-expressed LSP1.

Using *in vitro* adhesion assay and immunoblotting, we showed that endothelial cell-expressed LSP1 was phosphorylated only in the presence of adherent neutrophils or engagement of endothelial intercellular cell adhesion moelecule-1 (ICAM-1), but not cytokine or chemokine alone. Pharmacological inhibition of p38 MAPK by SB 203580, a selective MAPK inhibitor, significantly blunted the phosphorylation of endothelial LSP1. In endothelial cells, ICAM-1 engagement, *in vitro*, also caused a novel interaction between LSP1 and moesin which was mediated by p38 MAPK and Rho kinase. Absence of either LSP1 or moesin blunted the ICAM-1 engagement-induced endothelial permeability changes.

Using scratched wound healing and *in vitro* transmigration assay, we found that silencing of LSP1 reduced migratory behavior of endothelial cells. Selective deficiency of endothelial cell-expressed LSP1 also resulted in a marked reduction of neutrophil transendothelial migration and a subtle reduction in endothelial cell proliferation and neutrophil adhesion. The study demonstrates that LSP1-deficiency resulted in GATA-2-mediated selective reduction of endothelial cell-

expressed platelet endothelial cell adhesion molecule-1 (PECAM-1) that altered the endothelial cell functions.

Using murine primary endothelial cells and recombinant LSP1 expressed in mouse endothelial cell line (SVEC4-10EE2) cells and human embryonic kidney (HEK293T) cells, we demonstrate that LSP1 is post-translationally modified by small ubiquitin-like modifier 1 (SUMO1). Single lysine-to-alanine mutation at K270 and K318 of murine LSP1 prevented the SUMOylation of recombinant LSP1 in HEK293T cells. DeSUMOylation resulted in marked reduction in steady-state LSP1 levels. It is shown that reduced steady-state level of SUMOylation-deficient LSP1 was due to enhanced ubiquitination and subsequent rapid proteasomal degradation and that deSUMOylation of LSP1 impaired its translocation from nucleus to extranuclear compartments in endothelial cells in response to TNF-α stimulation.

This study confirms that adhesion-mediated phosphorylation is required for endothelial cell-expressed LSP1 functions whereas SUMOylation helps to maintain its functions by protecting LSP1 from proteasomal degradation and facilitating its translocation from nucleus to cytoskeleton in endothelial cells.

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DEDICATION

To my wife

Monjuara Mukta

For making me a better person each passing day and for helping me in achieving my goals. You mean a lot to me.

To my daughter

Yousra Hossain

For her precious smiles and affectionate hugs that help me relax in the end of tough, long days

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LIST OF ABBREVIATIONS

Ang1	Angiopoietin 1
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
CDC42	Cell division control protein 42
C-ERMAD	C-terminal ERM-associated domain
CNK	Connector enhancer of KSR
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCL5	Chemokine (C-X-C motif) ligand 5
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
EBP50	ERM-binding phosphoprotein 50
ECAM	Epithelial cell adhesion molecules
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ERM	Ezrin/Radixin/Moesin
ESL-1	E-selectin ligand-1
FERM	Four-point-one (4.1) ezrin, radixin, moesin
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GSK3	Glycogen synthase kinase 3
HCAM	Homing cell adhesion molecule
HECT	Homologous to E6AP Carboxy terminus
HGF	Hepatocyte growth factor
ICAM-1	Intercellular cell adhesion molecule-1
ICAM-2	Intercellular cell adhesion molecule-2

IGF-1	Insulin-like growth factor-1
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
JAM	Junctional adhesion molecule
JAMC	Junctional adhesion molecule C
KC	Keratinocyte-derived chemokine
KSR	Kinase suppressor of Ras
LFA-1	Lymphocyte function-associated antigen-1
LPAM	Lymphocyte Peyer's patch adhesion molecule
LSP1	Leukocyte-specific protein 1
LTB4	Leukotriene B4
Mac-1	Macrophage antigen-1
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MCP-1	Monocyte chemoattractant protein-1
Mdm2	Murine double minute 2
MEK1	Mitogen activated protein kinase kinase 1
NAD	Neutrophil actin dysfunction
NHERF	Na^+/H^+ exchanger regulatory factor
NRE	Negative regulatory element
p38 MAPK	p38 mitogen-activated protein kinase
PAMP	Pathogen-associated molecular pattern
PARK7	Parkinson's disease 7
PDGF	Platelet-derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule-1
Pgp-1	Phagocytic glycoprotein-1
PI3K	Phosphatidylinositol 3-kinase
PIAS	Protein inhibitor of activated STAT
PIP2	Phosphatidylinositol (4,5)-bisphosphate

PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PP2B	Protein phosphatase 2B
pRB	Retinoblastoma protein
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PTM	Post-translational modifications
Ran-GAP1	Ran-GTPase-activating protein 1
RING	Really interesting new gene
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SAE1/SAE2	SUMO activating enzyme 1/2
SENP	Sentrin/SUMO-specific proteases
SOD1	Superoxide dismutase 1
SUMO	Small ubiquitin-like modifiers
TBP	TATA-binding protein
TGF-β1	Transforming growth factor-β1
TNF-α	Tumor necrosis factor-α
Ubc9	Ubiquitin-conjugating 9
VAP1	Vascular adhesion protein 1
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4

1.0 INTRODUCTION

This thesis presents my studies on how endothelial cell-expressed leukocyte-specific protein 1 (LSP1) becomes phosphorylated and regulates endothelial cell functions, whether LSP1 is post-translationally modified by SUMOylation and whether this modification affects endothelial cell-expressed LSP1. Endothelial functions investigated include endothelial permeability, neutrophil adhesion, transendothelial migration and endothelial cell migration. Major mechanisms explored include LSP1-mediated transcriptional regulation of endothelial cell-expressed platelet-endothelial cell adhesion molecule-1 (PECAM-1) and LSP1-moesin interaction. Therefore, the introduction will begin with short discussions on features of acute inflammation such as endothelial permeability, the leukocyte recruitment cascade, adhesion molecules and ligands involved in different steps of leukocyte recruitment and the role of some these adhesion molecule-ligand interactions in the recruitment process. Post-translational modifications (PTMs) of proteins will be discussed briefly with an emphasis on phosphorylation and SUMOylation. Following brief background discussions on transcriptional regulation of protein expression, protein-protein interactions, LSP1 and ezrin/radixin/moesin (ERM) proteins, rationale and objectives of this set of studies will be highlighted.

1.1 Inflammation

Efficient removal of foreign invaders and harmful stimuli such as infectious pathogens, toxic chemicals and damaged tissues is central to survival of a living organism. The complex set of response to these invaders and stimuli through which an organism performs these functions is known as inflammation. This is the body's protective response against the infection and tissue damage that clears, dilutes, or walls-off the invaded pathogens and the injured tissues. It also initiates the healing and repair of the injury sites. Therefore, inflammation is crucial because in its absence wounds will never heal and infections will always go unchecked [1, 2].

However, components of this response that destroy and/or eliminate noxious stimuli has the ability to destroy or damage normal host tissues. Initial injurious stimulation is usually accompanied by normal beneficial reactions if the inflammatory response is finely balanced. However, the reaction can easily turn into a pathologic one if it is inappropriate such as when it is directed against self-antigens as in auto-immunity, harmless environmental antigens as in

hypersensitivity, too strong as in severe infection or prolonged when the causative agent resists the eradication [3].

Depending on its onset and duration, inflammation can be divided into either acute or chronic inflammation. Acute inflammation develops very quickly and is characterized by marked vasodilation, increased vascular permeability and neutrophil infiltration. On the other hand, chronic inflammation is the presence of an active inflammation for a prolonged duration (weeks to years) in which tissue destruction and repair take place simultaneously. In a chronic inflammatory site, the majority of the infiltrated leukocytes are mononuclear cells such as lymphocytes, macrophages, and plasma cells [3].

1.1.1 Vascular permeability

When acute inflammation sets in, volume of blood flow increases at the site of inflammation due to arteriolar vasodilation resulting in increased intravascular hydrostatic pressure. Plasma-rich fluid firstly moves from capillaries to the surrounding tissues due to this rising pressure. Very soon, rise in vascular permeability leads to loss of protein-rich fluid into the surrounding tissues increasing the osmotic pressure of the interstitium. Water and electrolytes keep moving into the surroundings in an attempt to balancing the intra- and extravascular osmotic pressure. The resulting fluid accumulation in the extravascular tissues is known as edema. Increased vascular permeability in acute inflammation, however, can be attributed to any one or a combination of the following mechanisms:

1. Formation of intercellular gaps in postcapillary venules due to endothelial cell contraction – this is a rapid and reversible response usually elicited by histamine, bradykinin, leukotrienes, and many other chemical mediators. Cytokine-induced endothelial cell retraction is a prolonged but slower process resulting from cytoskeletal rearrangement [4, 5]. Although all of these mediators disrupt endothelial barrier function by cytoskeletal rearrangement of the endothelial cells, the underlying mechanisms are completely different. For example, histamine regulates cell contraction by modulating endothelial calcium signaling, myosin light chain kinase and phosphorylation of junctional proteins whereas bradykinin's effect is mediated by endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) [4].

2. Direct injury to vascular endothelium by burns, certain infections or other severe injuries may result in immediate or sometimes delayed vascular leakage usually by causing endothelial cell detachment and necrosis.
3. Activated leukocytes may also damage the endothelium during adhesion and transmigration by secreting toxic mediators. Leukocyte-mediated endothelial cell injury often results in increased endothelial permeability by causing endothelial detachment [3, 6]. Leukocyte-endothelial cell interactions may also induce endothelial hypermeability by contracting endothelial cells in a Ca^{2+}-dependent manner [7].
4. Healing and repair of injured tissue requires the formation of new blood vessels, a process commonly known as angiogenesis. These vessel labyrinths remain leaky until the migrated, vessel forming endothelial cells form intercellular junctions via remodeling and maturation. Endothelial cells of these newly forming vessels express high level of receptors for angiogenic and vasoactive mediators, hence, are very leaky [3, 8].

1.1.2 Leukocyte recruitment

Leukocyte recruitment and vascular hypermeability are the hallmark signs of acute inflammation which occurs within minutes to hours [1]. During acute inflammation, leukocyte recruitment takes place in postcapillary venules where the hemodynamic shear forces is quite low. This is a tightly regulated multistep process of trafficking leukocytes from the flowing blood stream at the vasculature to the inflamed tissue which is fairly similar for different subsets of leukocytes in various inflammatory conditions [9].

Leukocyte recruitment is the result of sequential and often overlapping steps mediated by the interactions of adhesion molecules expressed on leukocytes and their ligands expressed on the surface of endothelial cells [10]. However, based on *in vivo* observations, it was first described as a two-step model consisting of leukocyte rolling followed by firm adhesion [11]. With the advent of science and modern technologies, the leukocyte recruitment paradigm has so far been updated dramatically (Figure 1.1). Although most of the recent investigations are based on neutrophil recruitment, other leukocytes also usually follow a similar multi-step cascade with some variations. According to the updated paradigm, recruitment starts with the capture of the free-flowing leukocytes to the surface of the endothelium commonly known as tethering which is followed by slow rolling along the endothelium. Following firm adhesion, leukocytes crawl along

the endothelium in search of suitable places for transendotheial migration. Then they migrate across the endothelium either transcellularly or paracellularly, and finally they migrate toward the site of infection or injury in the tissue.

1.1.2.1 Endothelial activation, leukocyte tethering and rolling

Endothelial cell activation is the prerequisite for leukocyte recruitment. Endothelium can be activated by the pattern recognition receptor (PRR)-mediated endothelial cell-pathogen interactions or by the inflammatory mediators (e.g., cytokines, leukotrienes and histamine) released from tissue resident cells or sentinel leukocytes when these cells come in contact with pathogens. This activation upregulates endothelial adhesion molecules [12, 13].

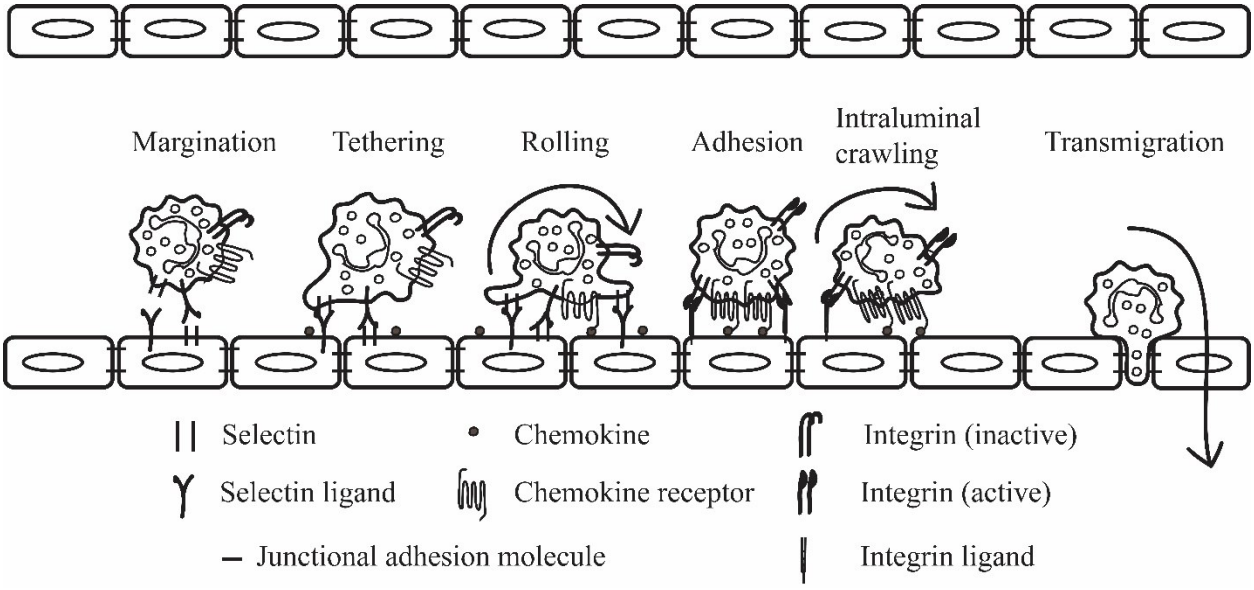

Figure 1.1: Sequential steps of neutrophil recruitment at post-capillary venule. Activated endothelium upregulates adhesion molecules on the luminal surface and drives neutrophil margination. Selectins are the first group of adhesion molecules that get upregulated during inflammation. The initial steps of neutrophil recruitment (tethering and rolling) are mostly selectin-dependent. Interaction of chemokines lining the luminal part of endothelium with their receptors on neutrophils induces conformational changes (active conformation) of neutrophil surface integrins via inside-out signaling. Therefore, the later steps (firm adhesion, crawling and transmigration) depend primarily on the interactions of integrin and their ligands. Crawling neutrophils follow the chemokine gradient along endothelium, which guides them to the preferential sites of transmigration (adapted from [14]).

In response to the inflammatory or infectious stimuli, endothelial cells upregulate selectins and then immunoglobulin-like cell adhesion molecules (CAMs such as ICAM-1 (intercellular cell

adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1)) on their surface. Weibel-Palade bodies contain stored P-selectins in endothelial cells. Upon activation, P-selectins are exocytosed from Weibel-Palade bodies to the surface of endothelial cells within minutes and P-selectins can be synthesized by the transcriptional and translational regulations and expressed on the endothelial cell surface within hours. However, E-selectin upregulation requires synthesis of new E-selectin molecules hence requires at least a few hours [9, 15]. Integrins are a family of adhesion molecules constitutively expressed on the surface of leukocytes. Upon leukocyte activation, integrins become activated via conformational changes and may also become upregulated in the number of expressed molecules. Integrins are transmembrane heterodimers consisting of an α-subunit and a β-subunit [16, 17]. In mammals, 24 different integrin heterodimers have been described which are formed by combining 18 different α and 8 different β subunits [18, 19]. Most notable integrins involved in leukocyte recruitment are: α_4 integrins (CD49d) such as $\alpha_4\beta_1$ (CD49d/CD29, also known as VLA-4–very late antigen-4) and $\alpha_4\beta_7$ (LPAM–lymphocyte Peyer's patch adhesion molecule) and the β_2 integrins, namely $\alpha_L\beta_2$ (LFA-1–lymphocyte function-associated antigen-1 also known as CD11a/CD18), $\alpha_M\beta_2$ (Mac-1–macrophage antigen-1 also known as CD11b/CD18), $\alpha_D\beta_2$ (CD11d/CD18) and αxβ2 (CD11c/CD18). Selectins on the surface of endothelium and their ligands specifically α_4 integrins and PSGL-1 (P-selectin glycoprotein ligand-1) on leukocytes mediate initial contact (tethering) between free-flowing leukocytes and endothelium *in vivo*. Subsequently, tethered leukocytes roll along the vessel wall before being arrested (Figure 1.1) [12, 20, 21]. Initial rolling is mediated mainly by the interaction between P-selectin and its ligand PSGL-1. E-selectin is vital for leukocyte slow rolling. Apart from PSGL-1, E-selectin also binds with ESL-1 (E-selectin ligand-1), CD44 also known as Hermes antigen, Pgp-1 (phagocytic glycoprotein-1) and HCAM (homing cell adhesion molecule). In addition to slow rolling, E-selectin-CD44 interaction also signals leukocytes to redistribute L-selectin (leukocyte-expressed selectin) on their surface [9, 22]. Leukocyte-expressed $\alpha_4\beta_1$ integrin binds to VCAM-1 while $\alpha_4\beta_7$ integrin interacts with both VCAM-1 and MAdCAM-1 (mucosal addressin cell adhesion molecule-1). Besides mediating β_2-ICAM-1 independent firm leukocyte adhesion, these interactions are capable mediating leukocyte rolling independent of selectins [23-25]. Rolling leukocytes may adhere firmly by activation-induced interactions of leukocyte integrins with

endothelial counter-receptors and transmigrate through the endothelium into the surrounding tissue [20, 21].

1.1.2.2 Leukocyte adhesion

In addition to adhesion molecules, activated endothelium also present chemokines such as chemokine (C-X-C motif) ligands 1, 2 and 5 (CXCL1, CXCL2 and CXCL5) on the surface. Positively charged chemokines are immobilized on the endothelial surface by binding to negatively charged heparan sulphates thereby creating a chemotactic gradient within the inflamed vessel. The contact between leukocytes and chemokines (on the endothelial surface) is facilitated by leukocyte rolling which is crucial for leukocyte activation. Leukocyte full activation is generally considered to be a two-step process which began with priming of leukocyte by pro-inflammatory cytokines or leukocyte-endothelial cell contact. Following initial priming, leukocytes get activated by their exposure to chemoattractants, growth factors or PAMPs (pathogen-associated molecular pattern) [14, 26]. Leukocytes, specifically neutrophils, constitutively express high level of β_2 integrins, i.e., LFA-1 and Mac-1 on their surface [14]. Chemokine binding activates the chemokine receptors (CXCR or CCR; which are G-protein coupled receptors) on the surface of rolling leukocytes results in an inside-out signaling and subsequent conformational changes of the cell surface-expressed integrins, e.g., LFA-1 and Mac-1. This conformational change (activation) increases the affinity of the integrins for their ligands including ICAMs as well as integrin valency (density of integrin heterodimers per unit area of plasma membrane) [27-30]. Enhanced integrin affinity is a prerequisite of initial leukocyte arrest (Figure 1.1) while increased valency strengthens adhesion. Clustering of activated integrins initiates the so-called integrin outside-in siganling. LFA-1 plays a more prominent role in the slow rolling and initial leukocyte arrest while Mac-1 comes into play during leukocyte firm adhesion and crawling along the endothelium which ultimately facilitates leukocyte extravasation [9, 30]. Among the endothelial adhesion molecules, ICAM-1 binds with both LFA-1 and Mac-1 and is hence considered as the most important endothelial adhesion molecule for leukocyte adhesion. Antibody-mediated ICAM-1 ligation and cross-linking are considered equivalent to leukocyte rolling and adhesion, respectively *in vitro*. On the other hand, VLA-4 and VCAM-1-mediated adhesion is also reported which is β_2 integrin and ICAM-1 independent.

1.1.2.3 Leukocyte crawling

Leukocytes are ready for transmigration during adhesion. However, for a large number of adherent leukocytes, transmigration does not occur right at the site of their firm adhesion. Many adherent leukocytes elongate themselves and continuously keep sending out pseudopods in search for a suitable spot for transmigration [31, 32]. Adherent leukocytes that are away from the endothelial cell-cell junction (the optimal transmigration site) actively crawl to the junction. Interactions between leukocyte-expressed Mac-1 and LFA-1 and endothelial cell-expressed ICAM-1 and ICAM-2 dictate intraluminal crawling (Figure 1.1) [32, 33]. Crawling leukocytes especially under flow maintain adhesion by breaking and simultaneously forming new bonds between adhesion molecules expressed on leukocytes and on the endothelium. Mechanotactic behavior of leukocytes plays a role in the process. Coordinated signaling involving cell division control protein 42 (CDC42); a major regulator of the organization of the actin cytoskeleton during leukocyte polarization and migration and VAV1 (a guanine exchange factor for the Rho-family GTPase Rac) is also believed to be important for leukocyte intraluminal crawling [14, 34]. Chemotactic gradient is not required for leukocyte crawling *in vivo* or *in vitro*, however, it helps direct the crawling leukocytes to the site of transendotehlial migration [14].

1.1.2.4 Leukocyte transmigration

For transmigration, leukocytes must cross two layers of barrier: first the endothelial barrier [35]. Transmigration is one of the most studied steps of leukocyte recruitment. A wide variety of cell adhesion molecules are known to be important for leukocyte transmigration. In addition to integrins and CAMs (ICAM-1, ICAM-2 and VCAM-1) that are necessary for transmigration, a number of junctional proteins such as platelet/endothelial cell adhesion molecule-1 (PECAM-1; also known as CD31), CD99, junctional adhesion molecules (JAMs), epithelial cell adhesion molecules (ECAM) play an important role in the process. Some other endothelial cell molecules including leukocyte-specific protein 1 (LSP1), poliovirus receptor and vascular adhesion protein 1 (VAP1) are also shown to be important for leukocyte transmigration, even though, the mechanism is not very clear [35-38]. Leukocytes take either paracellular (between endothelial cells; Figure 1.1) or transcelluar (through the endothelial cell) route to cross the endothelial barrier, although the latter is less prevalent. Paracellular transendothelial migration requires release of the junctional intercellular protein bonds. Probably, this is why, paracellular migration preferentially occurs at the tricellular corners where the junctional barrier is the least [39]. Interaction between

endothelial cell- and leukocyte-expressed adhesion molecules initiates many signaling pathways in the endothelial cells [10, 40, 41]. For example, ICAM-1 engagement-mediated elevated levels of intracellular Ca^{2+} activate myosin light chain kinase in endothelial cells resulting in the contraction of these cells. Endothelial cell contraction opens up the endothelial cell-cell adherens junctions leading to enhanced vascular permeability and leukocyte transmigration [9, 42, 43].

Endothelial cells form microvilli-like projections called “transmigratory cups” that move up the side of the leukocytes migrating transcellularly. *In vivo*, these projections go all the way up to the top of the leukocyte and was named as dome. However, the dome structure is always incomplete *in vitro*. These ICAM-1- and VCAM-1-rich projections form cup-like structures surrounding adherent leukocyte in a LFA-1- and VLA-4 (also known as $\alpha_4\beta_1$ integrin)-dependent manner, respectively [44, 45]. The F-actin–binding protein LSP1 is important for endothelial dome formation [37]. In this process, the transmigrating leukocyte never enters the intracellular compartment of the endothelial cells; the leukocyte is just covered and sealed away from the blood stream by the endothelium [14, 37].

Once passed through the endothelial layer, basement membrane imposes itself as the second, continuous and tougher barrier on the migrating leukocyte. The basement membrane is mainly composed of extracellular matrix proteins such as different vascular laminins and collagen type IV interconnected by glycoproteins such as nidogens and perlecan [9, 46]. Although the basement membrane is a continuous barrier, there are regions with thinner protein layers. These thinner regions express less collagen, laminin and nidogen 2, but not perlecan and usually overlap with interpericyte gaps [14, 47]. These are the areas where extravasating leukocytes would face the least resistance and therefore a well suited place for transmigration [47]. Homophilic PECAM-1 interaction during leukocyte transendothelial migration upregulates the surface expression of $\alpha_6\beta_1$ integrin on the migrated leukocytes. This integrin is a laminin receptor and another important role player in leukocyte extravasation across the basement membrane [48]. Moreover, extravasating leukocytes also secrete proteases such as matrix metalloproteinases and elastase. These proteases create holes in the basement membrane by degrading the matrix proteins thereby facilitates the escape of leukocytes from the vasculature [9, 49, 50].

1.1.2.5 Leukocyte extravascular tissue chemotaxis

Chemokine gradient across the vascular lumen is sufficient to move neutrophils out of the blood vessels. Once outside, these neutrophils need to quickly find a way to move against this

gradient in order to reach the site of injury or infection suggesting the presence of a new chemotactic gradient in the tissue capable of overriding the first gradient. Indeed, some recent studies suggest that different sets of chemoattractant molecules are released at different distance from the infection or injury sites. For example, the complement component C5a or bacteria-derived N-formyl-methionyl-leucyl-phenylalanine (fMLP) is dominant in the close proximity to the site of infection whereas CXCL8 (chemokine (C-X-C motif) ligand 8) and LTB4 (leukotriene B4) are predominatly released at intermediary sites. It is interesting to note that chemoattractants closer to the infection site override the signals from the chemoattractants present further away from the site of infection indicating the presence of a hierarchy of chemoattractants [51]. Based on these observations, chemoattractant molecules are functionally divided as 'end-target' and 'intermediate' chemoattractants. Neutrophil tissue chemotaxis was thought to be regulated solely by phosphatidylinositol 3-kinase (PI3K) pathway. It was largely accepted that the binding of chemokine to their receptors on the surface of neutrophils causes PI3K-mediated phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the leading edge of neutrophils [52], which favors their directional migration. Recent studies, however, support the presence of the hierarchy of intracellular signaling pathways as mentioned earlier. These studies also provide evidence that p38 mitogen-activated protein kinase (p38 MAPK) is crucial in end-target chemoattractants-induced signaling in neutrophils [14, 53].

Extravascular neutrophil chemotaxis is a very complex but relatively less studied step of the recruitement cascade. Neutrophil transmigration across endothelium may also affect tissue chemataxis specifically by affecting the surface expression of integrins in the transmigrating neutrophils [48]. Extravascular tissue locomotion also requires coordinated engagement and detachment of different neutrophil integrins with their ever-growing population of ligands in extravascular tissues. Morever, integrin cross-talk is also important for neutrophil tissue chemotaxis [54]. Because of the complex nature and insufficient knowledge of leukocyte tissue chemotaxis, more investigations are needed to uncover the molecular mechanisms of extravascular neutrophil chemotaxis.

1.1.2.6 Reverse neutrophil migration

It is widely accepted that transmigrated neutrophils die in the tissue and macrophages eat them up. However, in junctional adhesion molecule C (JAMC)-deficient mice, transmigrated neutrophils were shown to migrate back into circulation [10]. Similar observation was reported in

zebrafish embryo under sterile injury [11, 20]. Moreover, another report confirming the presence of reverse-transmigrating neutrophils in the blood of rheumatoid arthritis patients which were more resistant to apoptosis [21] poses a serious doubt on the generally accepted concept about death of all transmigrated neutrophils at the site of inflammation. The existence of reverse transmigration is unquestionable now, however, its implications are far from our understanding.

1.1.3 Endothelium in wound healing and tissue repair

Wound healing and tissue regeneration require the formation of new blood vessels from the existing ones, a process commonly known as angiogenesis. Migration of capillary endothelial cells is one of the essential steps of angiogenesis which consists of a series of events and is finely controlled by angiogenic factors [55]. Angiogenic agents stimulate endothelial cells to break their cell-cell contacts, proliferate, migrate into the perivascular space where they unite together by establishing their cell-cell association to form the vascular sprouts [8]. Maturation of these sprouts into blood vessels involves their continuous remodeling and recruitment of smooth muscle cells [56].

Many inflammatory disorders are characterized by excessive angiogenesis. Recruited leukocytes release a large number of angiogenic agents such as vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1), angiopoietin 1 (Ang1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), and many more [57, 58]. Leukocytes also help degrade barriers for migrating endothelial cells by releasing proteinases [59]. Angiogenesis is also turned off by hematopoietic cells via releasing or making some angiogenesis inhibitors [60, 61]. Inflammatory cells are also involved in adaptive arteriogenesis, a process of growing pre-existing collateral arterioles when the supply artery is occluded [62, 63]. In this process, high level of MCP-1 ensures large monocyte infiltrate and monocyte-mediated proteolytic remodeling of the vessel wall [8, 62] in favour of endothelial cell migration. Finally, TGF-β-, bFGF- and PDGF-B-mediated accelerated growth of endothelial cells and smooth muscles completes adaptive arteriogenesis to reduce the severity of the detrimental effects of blood vessel obstruction [8, 63].

1.2 Post-translational modification of proteins

Cellular functions in both eukaryotes and prokaryotes are largely dependent on gene expression that involves multiple complex processes such as transcription and translation. These processes are intricately regulated by cells for their growth and survival. In a cell, protein functions are finely regulated amidst ever-changing (internal or external) cellular conditions. After translation, functions of a protein are usually regulated by its interaction with other proteins and small molecules or by modifications (usually enzymatic in nature) of the protein molecule itself. One of the commonest mechanisms by which chemical structures, properties and functions of proteins are regulated is post-translational modification (PTM). PTM usually takes place during or after protein synthesis. It refers to the covalent and generally enzymatic modification of proteins necessary for the maturation of the modified proteins. PTM, in general, increases the functional diversity of the proteome and specifically crucial for cell signaling. So far, more than 300 different kinds of PTMs are known [64, 65] and over 200 of them are enzyme-mediated [65]; among them, the most notable are: phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, SUMOylation, lipidation and proteolysis. Phosphorylation, ubiquitination and SUMOylation will be discussed in details below.

1.2.1 Phosphorylation

Scientific community knew for a long time (as early as the 19th century) that phosphate could bind to proteins. In the old days, however, they thought that this was a common metabolic process of incorporating phosphorus as nutrient. Their belief was based on the fact that the then phosphate containing proteins were selectively available only in nutritious foods such as milk (casein) and egg yolk (phosvitin) [66]. It took until the late 1950's for the scientists to understand the fact that phosphoproteins are one of the key regulators of cellular life. The change in perception began with the discovery of the enzyme kinase and its role in transferring phosphate into another protein, a biological process called 'phosphorylation' [67]. In 1955, the stunning discovery by Fischer and Krebs [68], and Wosilait and Sutherland [69], revealed the functional regulation of phosphorylase by the addition or removal of phosphate. These studies first initiated the idea that reversible phosphorylation could control enzyme activity which was later proven to be true. For their extensive work and monumental contribution in establishing reversible phosphorylation-mediated regulation of enzymatic activity, Fischer and Krebs were awarded Nobel Prize in Physiology or Medicine in 1992.

At present, phosphorylation is considered to be the most prevalent reversible covalent PTM that regulates the functions of almost all different kinds of proteins such as enzymes, membrane channels, and other target proteins. In fact, almost every metabolic process in eukaryotes is regulated by phosphorylation of metabolic enzymes. Two groups of enzymes involved in this reversible process are namely protein kinases which are responsible for transferring a phosphoryl group from adenosine triphosphate (ATP) to the modified proteins and phosphatases which catalyze the hydrolytic removal of phosphoryl group from the phosphorylated proteins.

In eukaryotes, there are two classes of protein kinases, serine and threonine kinases and tyrosine kinases. Serine and threonine kinases mediate the transfer of the phosphoryl group to specific serine and threonine residues while tyrosine kinases mediate the transfer to specific tyrosine residues. Specificity of a protein kinase primarily depends on the amino acid sequence surrounding the serine/threonine or tyrosine phosphorylation sites known as phosphorylation motif. Because protein phosphorylation depends largely on the availability of phophoryl group donor ATP, it is usually restricted for intracellular proteins only. Reversible phosphorylation does not regulate extracellular proteins. The most exciting thing about phosphorylation is that the subtle change in protein conformation due to addition or removal of a phosphoryl group turns the modified protein on or off.

1.2.1.1 Phosphorylation in health and disease

Human genome codes more than 500 protein kinases and many protein phosphatases and about a third of total human proteins are phosphoproteins. These numbers are sufficient to guess that the imbalance in protein phosphorylation can be a cause or a consequence of some human diseases. Indeed, mutations of protein kinases and phosphatases are reported to cause a good number of diseases (Table 1.1). Interestingly, naturally occurring pathogens and toxins exert their effects by altering the phosphorylation states of proteins. A major virulence factor of *Yersinia* is a protein tyrosine phosphatase whereas, okadaic acid and microcystin are potent inhibitors of Type 1 and 2A protein phosphatases [70, 71]. These naturally occurring substances and their ability to modulate protein phosphorylation provide a nice opportunity of developing new therapeutics for diseases associated with altered protein phosphorylation. Some novel drugs that work by altering phosphorylation of proteins are listed below: cyclosporin is a lifesaving drug that made transplantation possible. It forms a complex with cyclophillin which inhibits calcineurin also

known as protein phosphatase 2B (PP2B) [1], an enzyme related to the Type 1 and 2A protein phosphatases.

Table 1.1: Diseases caused by mutations in particular protein kinases and phosphatases (used with permission [71]).

Disease	**Kinase/phosphatase**
Myotonic muscular dystrophy	Myotonin protein kinase
X-Linked agammaglobulinaemia	Bruton tyrosine kinase
Hirschsprung's disease	Ret2 kinase
Autosomal recessive SCID	Zap70 kinase
X-Linked SCID	Jak3 kinase
Craniosynostosis	FGF receptor kinase
Papillary renal cancer	Met receptor kinase
Chronic myelomonocytic leukemia	Tel-PDGF receptor kinase
Chronic myelogenous leukaemia	Abelson tyrosine kinase
Non-Hodgkins lymphoma	Alk kinase
Peutz–Jeghers syndrome	Lkb1 kinase
Coffin–Lowry syndrome	MAPKAP-K1b (RSK-2)
Ataxia-telangiectasia	Atm kinase
Li–Fraumeni syndrome	Chk2 kinase
Williams syndrome	Lim kinase-1
Leprechaunism, diabetes	Insulin receptor kinase
Wolff–Parkinson–White syndrome	AMP activated kinase
Wolcott–Rallison syndrome	eIF2A-kinase 3
X-Linked myotubular myopathy	MTM1 Tyr phosphatase

There are quite many protein kinase inhibitors under development that have the potential to fight diseases like cancer, chronic inflammatory disease and diabetes. Some examples include Erlotinib (CP 358774) that potently, selectively and reversibly inhibits epidermal growth factor receptor (EGFR) tyrosine kinase (useful in cancer treatment), SB 203580 that inhibits p38 MAPK (useful in chronic inflammation) and some glycogen synthase kinase 3 (GSK3) inhibitors to treat diabetes and stroke [71].

1.2.2 Ubiquitination

Ubiquitin is a small molecule (76-amino acid peptide), present in most cells, which is capable of being attached to a target protein in a process called ubiquitination. Ubiquitination is a post-translational modification of protein which determines whether the modified protein will be destined for degradation via 26S proteasome or enter into protein trafficking pathways [72].

Ubiquitination is a covalent modification of a protein which is carried out in a sequential manner by three distinct families of enzymes, namely, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). E1 enzyme initiates ubiquitination by ATP-dependent ubiquitin adenylation which is required for the activation of ubiquitin C-terminal glycine residue. Eventually, the activated ubiquitin forms a high energy thiolester bond with a thiol site of E1. Cysteine residue of the E2 active site receives ubiquitin from E1 through transacylation. E2 either directly mediates the conjugation of the C-terminus of the ubiquitin to the ε-amino group of a lysine residue of the target protein or forms an intermediate linkage between ubiquitin and E3 prior to transferring ubiquitin to the target protein (Figure 1.2) [73]s.

So far, there are only two E1 enzymes, ~40 E2 enzymes, and more than 600 E3 ligases reported in eukaryotes [74]. Although ubiquitination begins with activation by E1, E3 ligases provides substrate specificity to the activated ubiquitin [72]. Because E3 ligases are responsible for ubiquitin substrate recognition, there is a huge diversity of E3 ligases discovered and the growth of the family continues [73]. HECT (homologous to E6AP C-terminus) and RING (really interesting new gene) are the two well described families of ubiquitin ligases (E3). RING E3s facilitate ubiquitin transfer from E2 active site to substrate lysine(s) by acting as scaffolds whereas HECT E3s accept the ubiquitin from E2s and then transfer them to the substrate protein (Figure 1.2) [72]. Ubiquitination is classified as mono or polyubiquitination depending upon the nature and number of attached ubiquitin with its substrate. Modification of a protein by a single ubiquitin at a single site is called monoubiquitination whereas modification by multiple monomeric ubiquitins at several places on a single protein is defined as multi-monoubiquitination. However, attachment of ubiquitin chains containing at least four ubiquitin molecules, usually via 48 or 63 lysines on ubiquitin, to a protein is commonly known as polyubuquitination.

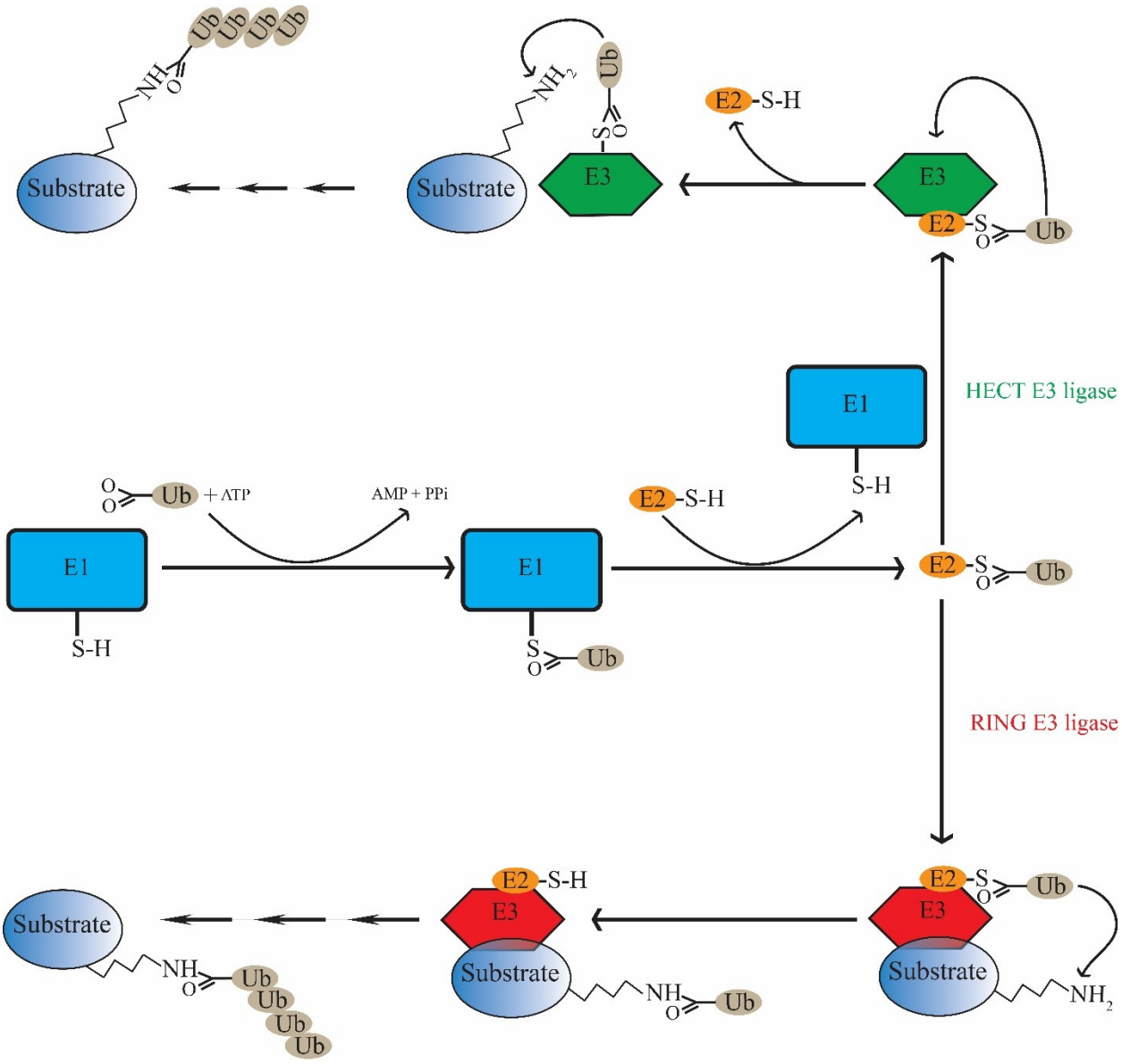

Figure 1.2: Schematic representation of the steps involved in ubiquitination.

The multistep ubiquitination process begins with ubiquitin activation, an ATP-dependent reaction which is immediately followed by the formation of thioester bond between the carboxyl terminus of a ubiquitin and a cysteine residue of a ubiquitin-activating enzyme (E1) and transfer of the ubiquitin to a specific cysteine residue of a ubiquitin-conjugating enzyme (E2). Ubiquitin can then be transferred from the E2 enzyme to the substrate either through E2-ubiquitin-E3 ligase complex (in case of RING E3 ligases) or through specific HECT E3 ligases. Final outcome is the formation of an isopeptide bond between ubiquitin C-terminus and specific lysine residue(s) of the target protein (used with permission from [75]).

Factors that dictate whether a substrate protein will undergo mono, multimono or polyubiquitination are largely unknown. Evidence suggests that a single substrate can follow all these modifications by different ligases and the same ligase can mono-, multimono-, or polyubiquitinate different substrates [76].

According to common understanding: polyubiquitination via lysine 48 leads the substrate protein to proteasomal degradation while polyubiquitination via lysine 63 linkage on ubiquitin follows non-proteasomal pathways, such as mediating DNA damage tolerance and protein trafficking [77]. Mono- and multimonoubiquitinations, on the other hand, are primarily believed to serve as signals for internalisation and entry into endocytic pathway [76, 78]. Monoubiquitination regulates a myriad of biological processes, such as transcription, DNA repair, histone modification and viral budding [79, 80]. However, recently, exceptions to these widely accepted functional changes due to ubiquitin modifications are rapidly growing in numbers.

1.2.3 SUMOylation

Small ubiquitin-like modifiers (SUMO) are a family of small proteins, reversibly but covalently attach to specific lysine residues of target proteins and modify their functions [81]. This process is known as SUMOylation, one of the relatively new PTMs. In vertebrates, there are four distinct members of the SUMO family (SUMO1, SUMO2, SUMO3 and SUMO4) [82]. Ran-GTPase-activating protein 1 (Ran-GAP1) is the first protein known to be modififed by SUMO. In 1997, Mahajan et al. identified two forms of Ran-GAP1, 70 kDa and 90 kDa in mammalian cells. They confirmed that the 90kDa form of RanGAP1 was modified by a novel small ubiquitin like modifier (SUMO) [83, 84].

SUMO2 and SUMO3 share about 98% sequence similarity, hence, are often referred as SUMO2/3. On the other hand, SUMO1 has only 50 % identical sequences as of SUMO2/3 [85, 86]. SUMO4, however is very similar to SUMO2/3, the major difference being substitution of glutamine at position 90 with proline. This substitution makes SUMO4 functionally distinct from the other SUMOs so that it is used by cells for modification of proteins only under stressful conditions such as starvation [87]. SUMO proteins are around 100 amino acid and about 12 kDa in molecular weight, however, the molecular weight may vary depending on the SUMO family member and the cell type where the protein is expressed.

Recent proteomic analysis using different SUMOs and various types of mammalian cells has led to the identification of a bulk of SUMO substrates. SUMOylation can regulate cellular

actions such as regulation of transcription, DNA repair and cell signalling by affecting a number of functions of the modified protein including localization, trafficking, protein-protein interactions, protein activity and protein stability [81, 88-94]. Dysregulation of protein SUMOylation was shown to be involved in many diseases such as cancer, diabetes, neurodegenerative diseases and many others suggesting an integral role of SUMOylation in regulating normal and abnormal cellular behavior [82].

1.2.3.1 SUMO conjugation, deSUMOylation and SUMO consensus motif

SUMO proteins are inactive precursors and requires C-terminus cleavage to expose its di-glycine motif, which is available for subsequent activation and conjugation. Sentrin/SUMO-specific proteases (SENP) are responsible for this cleavage [86]. In each conjugation cycle, SUMO is first activated in an ATP-dependent manner by the E1 activating enzymes (SUMO activating enzyme 1/2 (SAE1/SAE2)) and then passed to the active site of the only E2 conjugating enzyme Ubc9 (ubiquitin-conjugating 9). Finally, SUMO E3 ligases (protein inhibitor of activated STAT (PIAS) proteins) covalently attach SUMO to lysine residues of the target protein by forming isopeptide bonds between the terminal glycine residue of SUMO and the ε-amino group of a lysine residue(s) on the target protein [95-97]. Even though Ubc9 was shown to SUMOylate some SUMO substrates independently of any E3 enzymes, it is generally accepted that E3 increases SUMO transfer efficiency and substrate specificity of Ubc9 [98]. Like many other PTMs, SUMOylation is also a highly dynamic process and can be easily reversed by SUMO deconjugating enzymes such as the SENP enzymes [99] (Figure 1.3).

The SUMO consensus motif is a sequence of conserved residues around the modified lysine residue and is present in many SUMO substrates [100]. It is a tetrapeptide consensus motif ΨKxD/E, where Ψ is a large hydrophobic residue (such as Val, Ile, Leu, Met, or Phe). K is the lysine to which SUMO is conjugated, X is any amino acid and D/E is an acidic residue [82, 101]. About two-thirds of the known SUMO substrates contain this consensus motif [82, 101]. On the other hand, proteins lacking this motif can also undergo SUMOylation [102-104]. Moreover, lysine residues within this consensus motif are not always SUMOylated [95]. However, SUMOylation consensus motif is still used as a helpful tool for predicting SUMOylation sites.

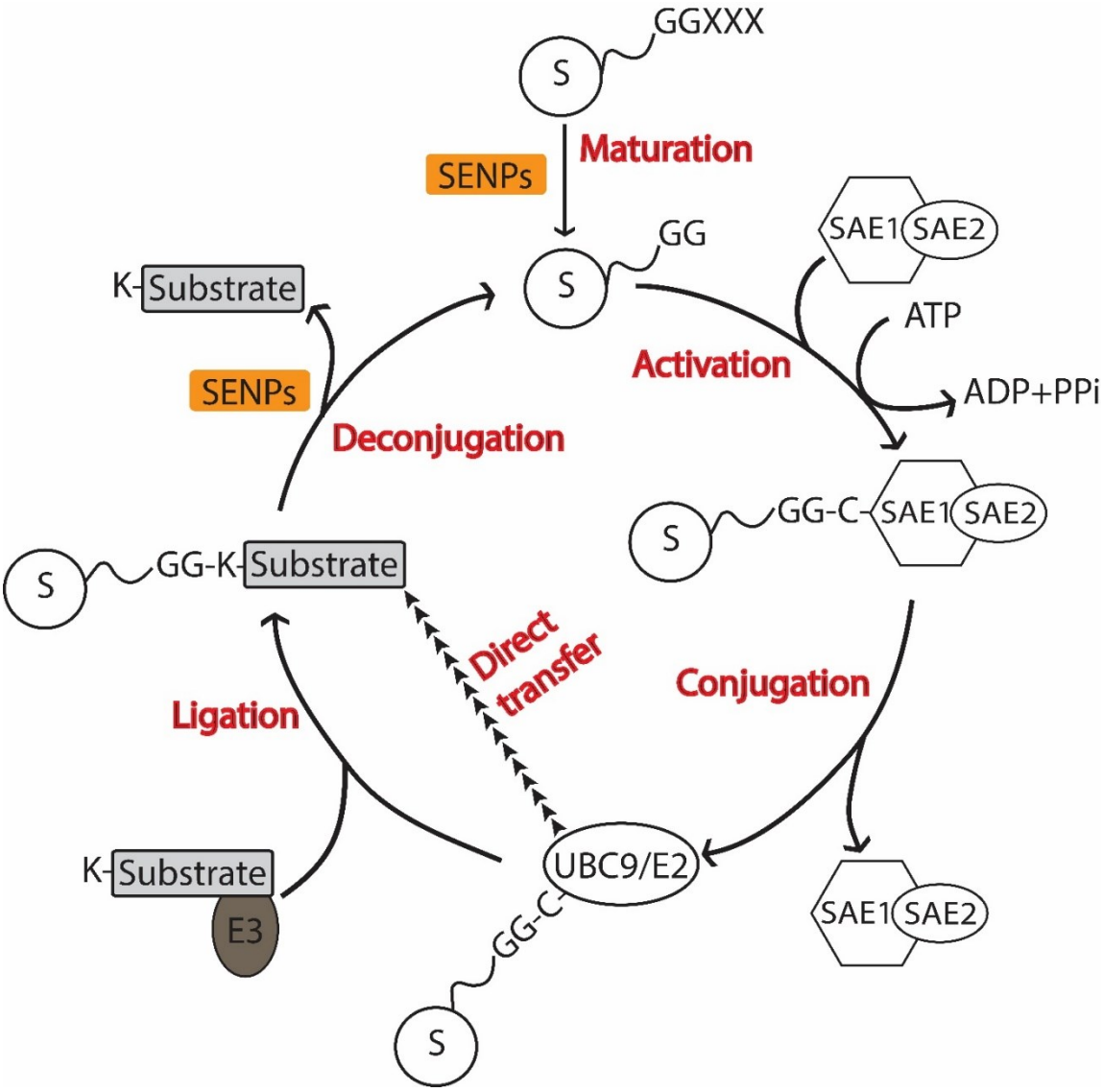

Figure 1.3: The SUMOylation cycle. Small ubiquitin-like modifier proteins (SUMOs) are synthesized as precursors and requires sentrin/SUMO-specific proteases (SENPs)-mediated cleavage of the SUMO C-terminus to reveal a Gly-Gly motif to enter into the SUMOylation cycle. This motif is essential for all subsequent steps of the SUMO cycle and mediates the direct linkage of SUMO to each enzyme and target molecule. Mature SUMO is activated by and covalently linked to a catalytic cysteine residue in the SUMO-activating enzyme 1 (SAE1–SAE2 heterodimer (E1 enzyme)). Activated SUMO is transferred to the catalytic cysteine residue of the SUMO-conjugating enzyme UBC9 (E2 enzyme). UBC9 catalyses the conjugation of SUMO to lysine residues in target proteins, either independently of or in concert with a SUMO E3 ligase, both of which determine target and lysine specificity. SENPs can cleave SUMO from target substrates (deconjugation), thus releasing an unmodified target and free SUMO capable of re-entering into the SUMOylation cycle (used with permission from [98, 105]).

1.2.3.2 SUMOylation *versus* ubiquitination

Ubiquitination and SUMOylation are two of the most important PTMs through which cells regulate the functions of many proteins. Even though they have some commonalities, they differ in many ways [54]. Both SUMO and ubiquitin are synthesized as inactive precursor proteins which are activated by the enzymatic (SENP) cleavage of the di-glycine motif from the C-terminal. They are similar in protein size, tertiary structure and a C-terminal di-glycine motif. Additionally, SUMOylation and ubiquitination requires the same set of enzymes, namely, E1 (activating enzyme), E2 (conjugating enzyme) and E3 ligases [86, 106]. While ubiquitination is well known for tagging the modified protein for degradation, SUMOylation, on the other hand, regulates the functions of modified proteins mainly by altering the stability, intracellular localization, nuclear trafficking, protein-protein interaction and transcription factor activity. Moreover, the ubiquitin pathway has been studied well for a long time and has a large number of E2 and E3 enzymes compared to SUMOylation [107].

1.2.3.3 SUMOylation and diseases

Like many other PTMs, SUMOylation plays an important role in favor of maintaining normal functions of many proteins while it may hinder the normal functions of many other proteins. This is why SUMOylation is critical for health and many diseases. In the recent past, several proteins that have key roles in pathogenesis of several human disease states, such as tau, Parkinson's disease 7 (PARK7) also known as DJ-1, ataxin-1, huntingtin and superoxide dismutase 1 (SOD1) were reported to be substrates of SUMO [86]. Many of these recent studies provide evidence on how SUMOylation could be involved in pathogenesis of human diseases, including cancer, neurodegenerative and heart diseases.

1.2.3.3.1 SUMOylation and cancer

Multiple lines of evidence suggest that the SUMOylation machinery plays an important role in different types of cancers. It was reported that hepatocellular carcinoma patients with elevated level of E1 (SUMO activating) enzyme had lower survival rates [108]. Ubc9, the only E2 conjugating enzyme for SUMOylation expression is upregulated in a number of human cancers. Ubc9 overexpression usually increases cancer cell growth [109, 110]. Upregulation of PIAS3, a SUMO E3 ligase was also observed in many cancer types [111]. Additionally, SUMOylation is known to modulate the functions of tumor suppressor proteins, such as p53, retinoblastoma protein (pRB), p63, p73, and murine double minute 2 (Mdm2) [112]. However, upregulation of the SUMO

protease SNEP1 was observed in prostate and thyroid cancers. This upregulation promotes the pathogenesis of cancer at least in the prostate [86, 113, 114]. These interesting findings make the relationship between cancer and SUMO pathway more complicated. Probably SUMOylation and deSUMOylation both play their distinct role in tumorigenesis of different tissues.

1.2.3.3.2 SUMOylation and neurodegenerative diseases

A number of proteins that play important role in the pathogenesis of several neurodegenerative diseases are known SUMO substrates. Most notable ones are huntingtin (Huntington's disease), ataxin-1 (spinocerebellar ataxia type 1), tau (Parkinson's and Alzheimer's disease), SOD1 (amyotrophic lateral sclerosis) and amyloid precursor protein (APP; Alzheimer's disease). However, the exact mechanism of how SUMOylation of these proteins relates the pathogenesis of the respective diseases is not completely understood.

SUMOylation was associated with increased stability of huntingtin while mutation of SUMOylation sites reduced aggregation of the mutant huntingtin. These findings indicate that SUMOylation possibly increases the levels of toxic intermediate poly-Q oligomers thereby increasing its ability to repress transcription [115].

1.3 Protein-protein interactions

Every cellular process practically depends on some sort of protein-protein interactions. Proteins rarely act alone and indeed protein-protein interactions are at the center of interatomics of virtually all living organisms. That is why, these interactions have enormous potential in the pathogenesis of diseases and to be targeted in the development of new therapeutic agents such as inhibitors of mouse double minute 2 (MDM2)–p53 interaction. These interactions can be divided into groups in several ways, such as homo- versus hetero-oligomers, stable *versus* transient interactions and covalent *versus* non-covalent interactions. Oligomers are macromolecular complexes. When a single protein forms a complex it is called homo-oligomer whereas protein complexes made up of different proteins are known as hetero-oligomers. In a stable interaction, proteins stay together for a long time and usually provide structural and functional support to the interacting proteins. On the other hand, transient interactions are reversible, brief and takes place only in specific cellular contexts such as stage of cell cycle, cell type and presence of other binding proteins. Most biochemical processes (signal transduction, transport across membranes, cellular metabolism, muscle contraction, etc.) are governed by transient protein-protein interactions. Protein-protein interactions usually affect protein functions in many ways including by changing

affinity and/or specificity for its substrate, by altering its kinetic properties, by inactivating a protein and even by opening up new binding sites [116].

Proteins are efficient in performing their functions (catalysis or transport) in the individual level. Crowded intracellular environment limits their effectiveness only to short-range. Sustaining life in this environment necessitates proteins to manage their long-range effectiveness via extremely organized and responsive protein networks called interactomes. Interactomes efficiently relay protein functions throughout the cell by means of protein-protein interactions [117-119]. Theoretically, many protein-protein interaction involved in disease pathogenesis is a potential drug target and ubiquitous nature of protein-protein interactions and related networks in cellular physiology provides ample opportunities for drug discovery [119, 120]. The interactome is not easy to solve because protein-protein interactions in cellular environment are very dynamic, some of these interactions takes place only in particular cellular contexts or at a certain stage of development and many of these interactions are transient [117]. For this reason, finding a protein-protein interaction is tricky even though of utmost importance. Below are the commonly used laboratory techniques to identify protein-protein interactions:

Physical methods to select and detect proteins that bind another protein

Protein affinity chromatography

Affinity blotting

Immunoprecipitation

Cross-linking

Library-based methods

Protein probing

Phage display

Antibody phage

Peptides on plasmids

Two-hybrid system

1.4 Transcriptional regulation of gene expression

Extensive genomic studies identified 30,000 genes in human genome [117]. It is interesting to note that these 30,000 genes are encoded in only about less than 2% of the genome. It is intriguing to wonder what exactly the remaining 98% of our DNA does. Although many mysteries remain about this majority of genome sequences, now we certainly know that some of those extra

sequences carry complex instructions for directing the intricate process of turning on or off gene transcription. These regulators of gene transcription also solve the mysteries about how and why specific stimuli lead to the synthesis of specific proteins in a cell type and why different cell types express very different proteins even though their DNA content is virtually the same [121]. Regulation of transcription is of utmost importance in gene expression. While post-transcriptional and translational regulation are well known, post-transcriptional steps of gene expression can take place automatically once the gene is transcribed into ribonucleic acids (RNA) [121]. Regulation of gene expression in eukaryotic cells is very intricate and requires combined efforts of several different types of transcription regulatory proteins. DNA methylation and packaging of DNA into chromatin make this tightly regulated process even more complex. Factors that usually regulate gene transcription are briefly discussed below.

1.4.1 Transcription factors

Transcription factors are proteins capable of controlling transcription of DNA to messenger RNA by binding to specific DNA sequences. They regulate transcription positively or negatively by themselves or by forming a complex with other transcription regulatory proteins via promoting or blocking RNA polymerase recruitment, respectively [122, 123].

1.4.2 Transcription regulatory DNA elements

Transcriptional regulation depends on the binding of the regulatory proteins to specific DNA sites which are usually organized into several distinct elements such as promoters, enhancers and silencers. [121].

1.4.2.1 Promoters

These cis-acting elements usually encompasses about 100 base pairs. Most promoters consist of transcriptional regulator binding site, a transcription initiation site and the TATA box [121, 123]. The TATA box is an AT-rich site located at 25 to 30 base pairs upstream of the transcription initiation site in higher eukaryotes. The TATA box is the binding site for the TATA-binding protein (TBP), so named because of its binding to the TATA box. Core promoters can be composite (containing both TATA and initiator (Inr) elements), TATA- or Inr-directed or null (neither of these elements) [124]. Many viral genes contain composite promoters. Some class II genes may contain Inr-directed promoter. However, most of class II genes contain TATA-directed

promoters. Transcription initiation for null promoters containing genes is not very precise because very often they have multiple initiation sites [125-127].

1.4.2.2 Enhancers

Unlike promoters, eukaryotic gene transcription can also be regulated by further distant DNA elements mostly upstream of the gene commonly known as enhancers or, sometimes, upstream activating sequences. Enhancers mostly contain multiple but same activator binding sites that are found immediately upstream of the promoter or binding sites for multiple different factors. Enhancers cannot drive transcription themselves, however, they increase promoter activity. Moreover, the presence of a cluster of binding sites enables enhancers to function independent of their location (upstream or downstream and up to 1,000,000 base pairs away from the transcription initiation site) and orientation relative to the promoter [121, 123].

1.4.2.3 Silencers

Silencers are DNA elements where repressors bind to repress promoter activity [128]. Mechanisms of repressor-mediated inhibition of transcription include prevention of transcription apparatus recruitment, inhibition of activator binding, and modification of chromatin structure [123, 129].

1.4.3 Transcription factor-mediated regulation of gene transcription

Upon binding to different DNA elements, transcription factors regulate transcription of a gene either positively or negatively. Transcription factors possess both DNA-binding and activation domains. Activation domains may directly interact with basal transcription complex or they may need coactivator molecules for this interaction. Binding of basic transcription complex to TATA box is sufficient to begin gene transcription, however, the speed is greatly increased by binding of other transcription factors to enhancers. On the other hand, transcriptional repressors negatively regulate transcription by blocking the activator binding site or forming a complex with activator that cannot bind to DNA or by organizing DNA into chromatin structure. Additionally, transcription is also regulated by controlling the level of transcription factor mainly in two ways: by modifying their synthesis and affecting their activity. Many transcription factors are only produced in specific cell types often in a stimulus-dependent process. For example, interleukin-6 (IL-6), usually secreted by some immune cells, induces the synthesis of NF-IL6β (a transcription factor) in cells expressing IL-6 receptors. This transcription factor ultimately stimulates the expression of its target genes in those cells. *De novo* synthesis of transcription factor is not always

necessary. In fact, in many instances, presynthesized transcription factors are turned on or off in response to appropriate stimuli. For example, in addition to NF-IL6β synthesis, IL-6 activates STAT-3 and NF-IL6, two transcription factors which are readily available in those cells [121, 123].

1.5 Leukocyte-specific protein 1 (LSP1)

Leukocyte-specific protein 1 (LSP1), was initially identified in normal and transformed B cells, in normal T lymphocytes but not (or in smaller amounts) in transformed T cells [130, 131] and later on, it was found in thymocytes, monocytes, macrophages, dendritic cells, and neutrophils [132-134]. LSP1 was first identified in murine and human lymphocytes hence given the name lymphocyte-specific protein 1. However, because of the subsequent discovery of the presence of this protein in all different kind of leukocytes, the scientific community renamed the protein as leukocyte-specific protein 1 [135]. Further investigations established that LSP1 was also expressed in mouse and human endothelial cells [136], and the amino acid sequences between leukocyte- and endothelial cell-expressed LSP1 were almost identical [137], indicating that LSP1 genes in leukocyte and endothelial cells have the same origin. [135].

LSP1 is an intracellular, Ca^{2+}- and F-actin-binding protein [138-141]. Human and mouse LSP1 proteins, which are 339 and 330 amino acid peptides respectively, share 67% identical sequences [131, 142, 143]. Ca^{2+} binding sites are identified in the N-terminus with mostly acidic residues and only 53% identical between the two species. In contrast, the C-terminus of LSP1, enriched in basic amino acids, holds two F-actin binding sites and is 85% identical between mouse and human LSP1 proteins [130, 138]. LSP1 transcription is regulated collectively by Ets, SP1 and C/EBP transcription factors. An Inr element determines a single transcription initiation site for LSP1 and the transcription is driven by a TATA-less promoter. Its expression in different cell types largely depends on the presence of anti-NRE (negative regulatory element), an anti-silencer. In the absence of anti-NRE, the silencer NRE inhibits the expression of LSP1 [144, 145].

1.5.1 Leukocyte-expressed *versus* endothelial cell-expressed LSP1

As opposed to predominant cytoplasmic distribution of LSP1 in neutrophils and B lymphocytes, endothelial cell-expressed LSP1 is mostly found in the nucleus, with small level of LSP1 found in F-actin-rich microfilaments [136, 138]. LSP1 translocates from nucleus to extranuclear compartments (predominantly to cytoskeleton) when endothelial cells are treated with TNF-α [37]. Conflicting findings in previous studies from different research groups showed that neutrophil-expressed LSP1 played a tissue-specific and either positive or negative regulatory role

on neutrophil adhesion, polarization, and migration [146, 147]. Using bone marrow transplanted chimeric mice, decreased transendothelial migration of neutrophils was observed in mice deficient in endothelial cell-expressed LSP1 in response to chemokine CXCL1 and cytokine IL-1β or TNF-α, effects attributed to the supportive role of endothelial-expressed LSP1 in neutrophil extravasation [136]. More recently, endothelial cell-expressed LSP1 was shown to regulate microvascular permeability by participating in endothelial dome formation during neutrophil transmigration [37].

During chemotaxis, LSP1 in neutrophils binds with F-actin in filopodia, lamellipodia, and membrane ruffles; while in B cells, LSP1 is recruited into anti-IgM-induced B cell receptor caps enriched in F-actin aggregates [139]. When binding F-actin during chemotaxis, LSP1 does not change the rate of actin polymerization, however, it rearranges polymerized F-actin into bundles [147, 148]. LSP1 is a major substrate of mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2) in the p38 MAPK pathway in neutrophils. MK2 phosphorylates LSP1 at serine 195 and 243 in murine neutrophils [143]. This signaling pathway is known to be important in neutrophil migration and chemotaxis in response to bacterial product formyl-methionyl-leucyl-phenylalanine (fMLP) and to CXC chemokine KC (keratinocyte-derived chemokine, CXCL1) [149-151]. LSP1 is also a substrate for protein kinase C (PKC) in B cells. PKC phosphorylates serine residues at 202 and 283 in the C-terminal of murine LSP1 [152, 153]. All these signaling kinases contribute to leukocyte migration. However, the mechanisms of endothelial cell-expressed LSP1 activation during leukocyte recruitment remain completely unknown. The complexity of cell-specific LSP1 signaling has, thus, promulgated discrepancies in the putative role of LSP1. Whether LSP1 modulates other steps of neutrophil recruitment such as intraluminal crawling and post-transendothelial extravascular chemotaxis is not known. Whether LSP1 has a role in endothelial cell functions such as endothelial cell proliferation and motility is yet to be studied.

1.5.2 LSP1 and human diseases

Neutrophil actin dysfunction with 47- and 89-kDa protein abnormalities (NAD47/89) is the first disease reported to be associated with abnormal LSP1 expression [154, 155]. Patients with NAD47/89 are susceptible to bacterial infections because their neutrophils exhibit defective migration, chemotaxis and phagocytosis. Neutrophils of these patients overexpress LSP1 with concomitant reduced expression of an 89 kDa protein, fail to increase actin polymerization in response to chemotactic factors and display numerous thin, hair-like, F-actin-rich filamentous

projections on their surfaces. Further studies revealed that indeed overexpression of LSP1 creates filamentous projections even in cells that do not constitutively express LSP1 and adversely affects motility of normally motile cells. However, it was also reported that a certain level of LSP1 is required for normal leukocyte motility and downregulation or upregulation negatively affects their motility [156-158].

A single nucleotide polymorphism in LSP1 gene was also reported to be associated with increased breast cancer risk [159, 160]. These reports are bioinformatics-based and predictive in nature. Whether LSP1 is indeed involved in the pathogenesis of breast cancer is yet to be studied. A recent report, however, demonstrated that LSP1-deficiency results in increased proliferation and migration of hepatocytes and hepatocellular carcinoma [161].

1.5.3 LSP1 binding partners

During chemotaxis of neutrophils, LSP1 co-localizes with F-actin in filopodia, lamellipodia, and membrane ruffles [146]. In B cells, LSP1 co-localizes with anti-IgM–induced B cell receptor caps enriched in F-actin aggregates [139]. LSP1 was found to directly interact with PKCβI but not with PKCα and βII. This interaction is required for extracellular signal-regulated kinases 2 (ERK2) activity, which is necessary for the survival of B cell lymphoma cells against anti-IgM–induced apoptosis [162]. This is a complex interaction involving a number of molecules e.g., ERK scaffold protein KSR (kinase suppressor of Ras), the ERK (MAPK), and mitogen-activated protein kinase kinase 1 (MEK1), the direct upstream activator of ERK. LSP1 functions as a targeting protein directing the KSR/MEK/ERK complex to a cytoskeletal location [163]. By interacting with cytoskeletal proteins, LSP1 interacts with DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) signalosome to facilitate virus transport into the proteasome of dendritic cells [164]. During carbohydrate signaling, scaffold proteins LSP1, KSR1 and CNK (connector enhancer of KSR) and the kinase Raf-1 constitutively forms a signalosome complex with DC-SIGN [165]. In macrophage, LSP1 interacts with myosin 1e and forms a complex in phagocytic cups where both members of this complex co-localize with actin [166]. Inhibition of these interactions largely impairs internalization of opsonized targets by impairing pseudopod formation. LSP1–myosin 1e interaction is crucial in Fcγ receptor–driven phagocytosis because this interaction regulates cytoskeleton remodeling [166].

1.6 Ezrin/Radixin/Moesin (ERM) proteins

Ezrin/Radixin/Moesin (ERM) proteins serve as linkers between the plasma membrane and the cortical F-actin cytoskeleton and they are very important for various cellular functions such as cell adhesion, cell motility and cell survival [167-171]. The amino terminal of the ERM proteins, also known as FERM (four-point-one ezrin, radixin, moesin) domain, mediates direct and indirect association with the plasma membrane whereas the carboxy terminal (C-ERMAD; C-terminal ERM-associated domain) domain is capable of binding to F-actin and intramolecular binding to the amino terminus. These two domains are linked by an α-helical linker. ERMs activity is regulated by an intramolecular association between the N-terminal and C-terminal domain that masks binding sites for partners and the proteins stay in a cytosolic 'dormant' state [172]. Activation of ERMs is a two-step process: a) binding of ERMs to the phosphatidylinositol 4,5-bisphosphate (PIP2) and other membrane proteins, such as CD44, ICAM-2 and EBP50 (ERM-binding phosphoprotein 50) [173, 174] making ERMs susceptible to phosphorylation at the conserved threonine residue, followed by b) phosphorylation at threonine residue by different kinases [175].

Upon activation, ERMs bind directly to the cytoplasmic tails of many membrane proteins via FERM domain, such as CD44, CD43 (also known as SPN) and intercellular adhesion molecules (ICAM-1/2/3) [174, 176]. They are also capable of binding to the related scaffolding proteins: ERM-binding phosphoprotein 50 (EBP50; also known as NHERF1; Na^+/H^+ exchanger regulatory factor 1) and NHE3 kinase A regulatory protein (E3KARP; also known as NHERF2; Na^+/H^+ exchanger regulatory factor 2). Both of them bind to multiple different membrane associated proteins themselves [177]. Thus, FERM domain of ERMs can bind to a large number of proteins directly or indirectly. Due to their ability to interact with cytoskeletal F-actin and diverse groups of other proteins, ERMs have the potential to bring cytoskeletal regulatory proteins in close apposition to the actin cytoskeleton. Though it has already been established that ERMs are capable of remodelling actin cytoskeleton, they appear to lack F-actin cross-linking or branch forming abilities [176]. Moreover, ERMs were reported to be abundantly present at both uropod of T-lympocyte and docking structure in endothelium. In both cases, they interact with adhesion receptors and are important for cell adhesion [178, 179]. In endothelial cells, moesin is the predominant (> 90%) ERM protein that regulates thrombin- and advanced glycation end products-induced endothelial hypermeability [180, 181].

1.7 Rationale for the study

LSP1 is an intracellular, Ca^{2+}- and F-actin binding protein [138-141] that plays an important role in leukocyte recruitment. Despite being near identical, leukocyte-expressed and endothelial cell-expressed LSP1 differ in many ways such as subcellular distribution (cytoplasmic *versus* nuclear) [136, 138] and functions (controversial role in leukocyte recruitment *versus* specific and limiting function in neutrophil transmigration and endothelial permeability). Information regarding how endothelial cell-expressed LSP1 functions is not available at all. Moreover, some intriguing albeit yet unexplored observations were recently reported about murine LSP1 in general and murine endothelial cell-expressed LSP1 in particular. Examples of such observations include appearance of LSP1 band in Western blotting (always > 50kDa as opposed to predicted m.w. of 37 kDa) [130, 141, 143, 182], characteristic distribution pattern of endothelial cell-expressed LSP1 and nucleus-to-extranuclear translocation upon TNF-α-treatment. Although PTMs often result in uplift of molecular weight [81, 183], compartmentalization and translocation of many proteins [88, 184, 185], they remain completely unexplored for endothelial cell-expressed LSP1. It is not clear whether endothelial cell-expressed LSP1 is activated by and act in the same singling pathways as leukocyte-expressed LSP1. Striking differences between leukocyte-expressed and endothelial cell-expressed LSP1 and characteristic features of endothelial cell-expressed LSP1 prompted us to speculate that murine LSP1, specifically the endothelial cell-expressed LSP1, is modified post-translationally and mechanism of action of these two versions of LSP1 may vary significantly. In addition to unraveling the role of endothelial cell-expressed LSP1 in leukocyte recruitment and endothelial cell functions, the study of molecular mechanism of this protein may reveal potential and hopefully specific target for novel anti-inflammatory therapeutics.

1.8 Hypothesis

Post-translational modifications, specifically SUMOylation/ubiquitination and phosphorylation, regulate the functions and characteristic properties of endothelial cell-expressed LSP1.

1.9 Objectives

Specific objectives of the investigations presented in this thesis are:

Specific objective 1: To elucidate the mechanism of phosphorylation of endothelial cell-expressed LSP1 with a view to understanding the significance of LSP1 activation in endothelium.

1a) To determine the level of LSP1 phosphorylation in SVEC4-10EE2 cells in the presence of various types of stimuli by Western blotting.

1b) To examine whether phosphorylated LSP1 is associated with cytoskeleton-binding proteins and regulate permeability changes in endothelial cells by co-immunoprecipitation followed Western blotting and Transwell permeability assay.

Specific objective 2: To assess the role of endothelial cell-expressed LSP1 in regulating other nuclear proteins and associated endothelial cell functions.

2a) To evaluate the role of LSP1 on GATA-2-dependent PECAM-1 expression in endothelial cells by Western blotting, RT-qPCR and confocal microscopy.

2b) To determine the effect of LSP1-mediated altered PECAM-1 expression on endothelial cell motility and migration by Scratch Wound Healing assay and Transwell migration assay.

Specific objective 3: To explore post-translational modification of endothelial cell-expressed LSP1 by SUMO1 and the consequences of this modification.

3a) To study whether endothelial cell-expressed LSP1 is modified by SUMO1 by coexpression, His pull-down, immunoblotting and confocal microscopy.

3b) To observe the effect of LSP1 SUMOylation on its distribution and stability using subcellular fractionation, cycloheximide chase assay and immunoblotting.

2.0 MATERIALS AND METHODS

2.1 Mice

Lsp1$^{-/-}$ mice on the 129/SvJ background were generated by homologous recombination by Jongstra-Bilen and colleagues [148]; both mouse strains were then transferred to the University of Saskatchewan. *Lsp1*$^{-/-}$ (knock-out; KO) mice were crossbred with *Lsp1*$^{+/+}$ (wild-type; WT) mice to generate heterozygotes and homozygotes (F2). The *Lsp1*$^{-/-}$ (KO) and *Lsp1*$^{+/+}$ (WT) homozygotes were confirmed by genotyping. Mice of these two genotypes were bred to obtain age- and sex-matched controls. Five- to 7-day-old and 8- to 16-wk-old mice were used in the experiments. The study was carried out with the approval of animal protocols from the University Committee on Animal Care and Supply at the University of Saskatchewan and following the standards of the Canadian Association of Animal Care. All surgeries were performed under ketamine-xylazine anesthesia, as previously described [136], and all efforts were made to minimize animal suffering. Bone marrow chimeric mice were generated according to a previously described protocol [136] and were housed in specific pathogen-free facilities for 6–8 wks to allow full humoral reconstitution before use in experiments. Bone marrows from WT mice transplanted into WT and KO mice are designated as WT→WT and WT→KO mice, and bone marrows from KO mice transplanted into WT and KO mice are designated as KO→WT and KO→KO mice, respectively. Chimeric mice generated according to this protocol were confirmed to have ~99% of leukocytes from donor mice [186].

2.2 DNA constructs

Mouse pCMV-SPORT6-His-LSP1, pCMV-SPORT6-Ubc9, pCMV-SPORT6-SUMO1, and pCMV-SPORT6-SENP1 plasmids were purchased from Thermo Scientific. HA-ubiquitin (Addgene plasmid # 18712) and pEGFP-moesin plasmids (Addgene plasmid # 20671) were purchased from Addgene, Cambridge, MA, USA [187]. All of these constructs were amplified in *Escherichia coli* DH5α and extracted using Midi Plasmid Kit (Qiagen, Toronto, ON). Mutations of potential SUMOylation sites of LSP1 lysine (K) residues at K270, K318 or K321 were introduced by performing one-step mutation PCR (Life Technologies Inc., Burlington, ON, Canada) on mouse pCMV-SPORT6-His-LSP1, using primers containing the respective lysine (K) to alanine (A) mutations as following:

LSP1^{K270A} forward: AGTCAGTCTGCTTCTGCGACACCCTCCTGCCAG

LSP1^{K270A} reverse: CTGGCAGGAGGGTGTCGCAGAAGCAGACTGACT

LSP1^{K318A} forward: GCCACTGGACATGGGGCGTACGAGAAAGTACT

LSP1^{K318A} reverse: AGTACTTTCTCGTACGCCCCATGTCCAGTGGC

LSP1^{K321A} forward: CATGGGAAGTACGAGGCAGTACTTGTGGATGAGGG

LSP1^{K321A} reverse: CCCTCATCCACAAGTACTGCCTCGTACTTCCCATG

2.3 Cell culture

2.3.1 HEK 293T and SVEC4-10EE2 cell (ATCC) culture

Frozen human embryonic kidney 293T (HEK 293T; ATCC, Manassas, VA) cells and SVEC4-10EE2 (ATCC, Manassas, VA), a murine microvascular endothelial cell line (EE2) cells (cryovial containing ~6 x 10^6 cells in 1 ml) in 5% DMSO and 10% fetal bovine serum (Hyclone, UT, USA) containing Dulbecco's modified Eagle's medium (DMEM; Cellgro, VA, USA) were rapidly thawed at 37°C, added to 10 ml DMEM and spun at 200×g for 3 minutes. The supernatant was removed and the cell pellet was resuspended in DMEM supplemented with 10% fetal bovine serum, penicinllin (100 units/ml; Amresco, OH, USA) and streptomycin (100 μg/ml; Amresco, OH, USA) and plated in 100-mm cell culture dishes. Cells were cultured in a 5% CO_2 incubator at 37°C and passaged and replated prior to reaching confluence.

2.3.2 Murine endothelial cell isolation and culture

Murine microvascular endothelial cells were isolated from the lungs (LVEC) or hearts (HVEC) of 5-7-days-old *Lsp1*$^{-/-}$ (KO) or 129/SvJ (WT) mouse pups. After isoflurane anesthesia, pups were dipped into 70% ethanol and decapitated. Hearts and lower lobes of the lungs were collected, minced into smallest possible pieces and digested in collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C. Cells were dissociated from the digested tissue by a 50-um cell strainer. Endothelial cells were then immunomagnetically isolated utilizing rat-anti-ICAM-2 (CD102) primary antibody (clone 3C4, BD Pharmingen, Quebec City, QC, Canada) and magnetic beads-conjugated anti-rat IgG secondary antibody (Miltenyi Biotec Inc., Cornerstone Court East, San Diego, CA, USA), seeded in laminin-coated 6-well plates or 35-mm petri dishes, and cultured in microvascular endothelial cell culture medium (EBM-2) supplemented with the EGM-2 MV BulletKit (Lonza, Mississauga, ON, Canada). Contaminating dead leukocytes were carefully washed off on the very next day and medium replacement was continued every other day. After

the cells reached confluency, they were passaged only once on laminin-coated 24-well plates, 6-well plates or glass cover slips before being used in respective experiments (usually within 15 days of isolation).

2.4 HEK 293T and EE2 cell transfection

HEK 293T or EE2 cells were cultured in 2 ml of DMEM supplemented with 10% FBS and penicillin/streptomycin in 6-well plates. All transfections were performed when the cells reached 70% confluency. Shortly before transfection, the media was replaced with 1 ml of antibiotic and serum-free DMEM. For gene silencing, siRNA targeting genes of interest and scrambled RNA. For overexpression studies, plasmid DNA containing the cDNA of proteins of interest and empty vector were diluted in sterile Eppendorf tube containing basic DMEM without antibiotics or serum (100 µl). In a separate Eppendorf tube, siRNA transfection reagent (Santa Cruz) for siRNA transfection or Lipofectamine 2000 (Invitrogen) for plasmid DNA transfection was also diluted in 100-µl basic DMEM and subsequently added to the tube containing the diluted cDNA within 5 minutes. The mixture (200 µl) was gently mixed and allowed to sit for ~30 minutes at room temperature for siRNA-transfection reagent or plasmid-Lipofectamine complexes to form before adding the mixture drop-wise to the well of a 6-well plate. After 4 hours of incubation in a cell culture incubator, 1ml of fresh DMEM with antibiotics and 20% FBS was added to each well of the 6-well plate. Cells were allowed to grow for 12–48 hours after transfection. Transfection efficiency of expression plasmids was ~70% in HEK 293T cells and 40-50% in EE2 cells as determined by expressing GFP-tagged plasmids in the respective cells. Transfection (determined by imaging of fluorescently labeled control siRNA) and knockdown efficiency (determined by RT-qPCR and western blotting followed by densitometric analysis of the respective protein bands) of the siRNAs used were in the range of 45-60% and 50-70%, respectively.

2.5 Isolation of murine neutrophils

2.5.1 Bone marrow neutrophils

Femurs and tibias from mice were dissected, and the marrow was flushed with ice-cold Ca^{2+}- and Mg^{2+}-free PBS solution. Bone marrow cells were separated by gentle mixing with a transfer pipette. Neutrophils were isolated using three-step Percoll (GE Healthcare, Uppsala, Sweden) gradient (72%, 64%, and 52%) centrifugation at 1,060×g for 30 min, as described previously [55]. This procedure yielded 80-90% morphologically mature neutrophils. These

neutrophils were stimulated with chemoattractant and used in *in vitro* adhesion and transmigration assays.

2.5.2 Peritoneal lavage neutrophils

Acute mouse peritonitis was induced to obtain emigrated neutrophils 4 hours after an i.p. injection of 0.5 μg macrophage inflammatory protein-2 (MIP-2, also known as CXC chemokine CXCL2; R & D Systems, Burlington, ON, Canada). The peritoneal cavity was then lavaged and the harvested cells (≥ 95% neutrophils) were briefly centrifuged before resuspension in ice-cold PBS containing 0.5% BSA.

2.6 Flow cytometry

Neutrophils transmigrated into the peritoneal cavity were collected by peritoneal lavage and integrin expression was determined as described previously [65] using the following FITC-conjugated fluorescent antibodies and the respective isotype controls (purchased from eBioscience, San Diego, CA) against α_2 (CD49b, 1:250 dilution, clone HMa2), α_4 (CD49d, 1:200 dilution, clone R1-2), α_5 (CD49e, 1:200 dilution, clone HMa5-1), α_6 (CD49f, 1:100 dilution, clone GoH3) and β_1 (CD29, 1:100 dilution, clone HMb1-1).

PECAM-1 expression in murine peripheral blood neutrophils was determined following a previously reported protocol [73] with minor modification. Mouse blood was collected by cardiac puncture in a heparinized syringe. Blood sample (100 μl) was incubated with FITC-conjugated anti-mouse PECAM-1 (CD31) mAb (1:100 dilution, clone 390, eBioscience) for 30 min at room temperature. The erythrocytes were lysed using a lysis buffer (Beckman Coulter, Mississauga, Ontario, Canada). The cells were then washed and analyzed by flow cytometry. For FACS analysis, neutrophils were gated on a linearly plotted forward scatter-side scatter dot plot.

2.7 *In vitro* adhesion assay

Freshly isolated mouse bone marrow neutrophils were stimulated for 5 min with MIP-2 (CXCL2), 100 nM; R & D Systems], keratinocyte chemokine (KC, also known as CXC chemokine CXCL1, 100 nM; Peprotech, Rocky Hill, NJ, USA), or thapsigargin (100 nM; Sigma, St. Louis, MO, USA). The neutrophils were allowed to adhere onto confluent monolayers of murine primary endothelial cells or SVEC4-10EE2 (EE2) cells, a mouse endothelial cell line in 24-well plates. Where indicated, unstimulated neutrophils were allowed to adhere onto tumor necrosis factor-α (TNF-α)-stimulated (20 ng/ml for 4 hours; R & D Systems) endothelial cells. Unlike MIP-2- or

KC-stimulated neutrophils, thapsigargin-treated neutrophils were subsequently washed twice in ice-cold PBS before they were allowed to adhere onto endothelial cells. The ratios of the number of neutrophils to endothelial cells were 0:1, 0.5:1, 1:1, 2:1, and 5:1. Where indicated, the p38 MAPK inhibitor SB-203580 (10 μM; EMD Millipore, Billerica, MA, USA) or a PKC inhibitor [staurosporine (100 nM; Selleckchem, Houston, TX, USA), sotrastaurin (5 μM; Selleckchem), or Gö-6983 (1 μM; Tocris, Bristol, UK)] was added during the assay. Unbound cells were removed by two washes with prewarmed (37°C) PBS, and the adherent neutrophils were stained using a three-step staining set (Richard-Allan Scientific, Kalamazoo, MI, USA), counted microscopically in triplicates, and averaged from five different fields of view (325 μm^2/field of view). To assess the role of endothelial LSP1, neutrophils from WT mice were placed on primary endothelial cells from KO or WT mice. To study phosphorylation of LSP1 in endothelial cells, only KO neutrophils were used in all experiments to rule out the influence of neutrophil LSP1.

To exclude the possibility of endothelial LSP1 activation by soluble mediators released from activated neutrophils or endothelial cells during neutrophil adhesion, endothelial cells were additionally treated with conditioned medium. Freshly isolated bone marrow neutrophils were stimulated with MIP-2 (100 nM for 10 min, 37°C) and then allowed to adhere to endothelial cells at 37°C for 30 min before centrifugation and collection of the supernatant (conditioned medium). Another confluent monolayer of endothelial cells was treated with this conditioned medium for 30 min at 37°C before endothelial phosphorylated LSP1 was detected.

2.8 *In vitro* neutrophil transendothelial migration assay

Murine microvascular EE2 endothelial cell line cells were cultured onto tissue culture-treated polycarbonate transwell inserts (3 μm pore size, Corning, Corning, NY, USA) until 85% confluence. After endothelial cells were treated with or without GATA-2-specific or non-targeting silencing RNA for 24 h, neutrophil transendothelial migration was examined as previously described [87, 95] with minor modification. In brief, freshly isolated murine neutrophils were suspended at 5×10^6 cells/ml in DMEM medium supplemented with 5% FBS. Neutrophils in suspension (1×10^6 cells) and chemoattractant (CXCL2; 200 ng/ml) were added to the top inserts and bottom wells, respectively. Neutrophils were allowed to migrate for 2 h at 37°C in 5% CO_2. Then, 60 μL of 0.5 M EDTA was added to the bottom chamber, and the plate was incubated for 10–15 min at 4°C. Inserts were removed from the well, and the total number of transmigrated cells in each bottom well was counted using a hemocytometer.

2.9 *In vitro* endothelial cell migration assay

Both non-directional lateral and chemoattractant-induced directional migration of endothelial cells were analyzed using the following *in vitro* assay systems:

2.9.1 Scratch wound healing assay

EE2 cells were grown in 6-well plates and treated with LSP1 or GATA-2-specific or non-targeting siRNA at 65% confluence. They were grown till 100% confluence and the monolayer was scraped in a straight line with a 1000 µl pipet tip to introduce a longitudinal scratch. Cell monolayers were washed with pre-warmed PBS and growth medium to remove the cellular debris and to get a smooth-edged scratch. Culture plates were marked properly at close proximity to the scratch imaging points when the images were acquired for the zero time point. These marks helped in obtaining the same field of view during image acquisition at indicated time points. All the images were acquired using an Olympus 1X51 inverted microscope (Olympus America, Center Valley, PA, USA) and analyzed quantitatively by ImageJ software (http://rsb.info.nih.gov/ij/). Endothelial cell migration was measured by comparing the distances (µm) between one side of scratch and the other for all the treatment groups at indicated intervals [188, 189].

2.9.2 Transwell migration assay

Bottom surfaces of the tissue culture-treated polycarbonate transwell inserts (3 µm pore size, Corning, Corning, NY) were coated with laminin overnight. SVEC4-10EE2 cells, treated with or without silencing RNA targeting LSP1, GATA-2 or non-targeting silencing RNA for > 24 h were trypsinized and suspended at 2×10^6 cells/ml in serum-free DMEM medium. Endothelial cell suspension (100 µl) was added to the upper chamber while 600-µl serum free DMEM medium was added to the bottom chamber. Cells were allowed to adhere onto the top surfaces of transwell inserts for 10 minutes. FBS (2% of the total volume) was added at the bottom chamber to induce cell migration and the cells were allowed to migrate along the FBS gradient for 2 h at 37°C in 5% CO2. Inserts were removed from the well, the remaining cell suspension from the top chamber was carefully removed, placed into new wells containing trypsin solution (0.025%) and incubated for 30 minutes at 37°C. Transmigrated cells were completely dislodged from the insert membrane by putting plate on a shaker several times for about a minute during incubation. Inserts were removed from the well, and the total number of transmigrated cells in each bottom well was counted using a hemocytometer [190-192].

2.10 Endothelial cell proliferation assay

Endothelial cell proliferation was evaluated by counting cells in a hemocytometer under a bright-field microscope [193]. SVEC4-10EE2 cells at 70% confluence were treated with or without silencing RNA targeting LSP1, moesin, GATA-2 or non-targeting silencing RNA for 12 hours. Then, these cells were trypsinized and seeded at 5×10^4 cells/well in 60 mm petri dishes and allowed to grow for 72 hours. Endothelial cells were then washed with PBS, trypsinized and suspended in medium for counting in a hemocytometer [194].

2.11 *In vitro* endothelial permeability assay

Endothelial permeability was determined by measuring FITC-albumin flux across confluent EE2 cell monolayers cultured on the luminal (upper) chamber of a Transwell system as described earlier [195]. EE2 cells were grown in cell culture-treated and laminin-coated 6.5 mm polycarbonate Transwell inserts (3 μm pore size, Corning, Corning, NY) until 70% confluence. Cells were then treated with or without silencing RNA targeting LSP1, moesin, GATA-2 or non-targeting silencing RNA and allowed to grow until confluence (> 24 h). Once the monolayer become uniformly confluent, ICAM-1 was cross-linked (on LSP1- or moesin-silenced cells) after 4 hours of TNF-α (20 ng/ml) treatment or neutrophils were allowed to transmigrate along a MIP-2 gradient in a Transwell system. Uniform confluency of endothelial monolayers was established by observing some stained control inserts under microscope. FITC-albumin (200 μg/ml) was added to the luminal chamber in 100 μl DMEM medium. After 1 or 2 hours, 100 μl samples were removed from both the luminal and abluminal chambers and their fluorescence was quantified using a Fluoroskan Ascent plate reader (Thermo Scientific). The excitation and emission wavelengths were 485 and 520 nm, respectively. Permeability index (%) was calculated using the following formula: ((mean lower chamber intensity – background) / (mean upper chamber intensity – background)) × 100 [37].

2.12 Cycloheximide chase assay

Cycloheximide chase assay was performed according to a previously reported protocol [196] with some minor modifications. HEK 293T cells were co-transfected with 2 μg of His-LSP1 or His-LSP1^{K270A} and 2 μg of SUMO1. Twelve hours after transfection, cells were treated with 100 μg/ml cycloheximide (Sigma-Aldrich, Oakville, ON, Canada) and then chased at 37°C for 0 to 12 h. At the appropriate chase time, cells were rinsed with ice-cold PBS and lysed in Ni-NTA

lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.05% Tween, pH 8.0) containing 10 mM imidazole supplemented with protease inhibitor cocktail (Sigma-Aldrich) on ice. His-tagged proteins were pulled-down using Ni-NTA slurry, resolved by SDS-PAGE and immunoblotted with anti-LSP1 antibody. The detected bands were then quantified using ImageJ software (v 1.47; http://rsbweb.nih.gov/ij). Relative protein quantity values for each time point were used for linear regression analysis, plotted as linear fits using origin software (OriginLab Corporation, Northampton, MA) and half-life values were calculated using the slopes obtained from the straight line equations for each fitted curve.

2.13 Proteasome inhibition and *in vitro* ubiquitination assay

HEK 293T cells were co-transfected with His-LSP1 and SUMO1; His-LSP1, SUMO1 and SENP1 or His-LSP1K270A and SUMO1. After 12 hours, cells were treated with proteasome inhibitor MG132 (10 μM) for 12 h, lysed and proteins were immunoblotted with an anti-LSP1 antibody. For the *in vitro* ubiquitination assay, HEK 293T cells were co-transfected with His-LSP1, SUMO1 and HA-Ub; His-LSP1, SUMO1, SENP1 and HA-Ub or His-LSP1K270A, SUMO1, HA-Ub in the presence or absence of proteasome inhibitor, MG132 (10 μM; 12 h). At the appropriate time, cells were rinsed with ice-cold PBS and lysed in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.05% Tween, pH 8.0) containing 10 mM imidazole supplemented with protease inhibitor cocktail (Sigma-Aldrich Canada Co., Oakville, ON) on ice. His-tagged proteins were pulled-down using Ni-NTA slurry, resolved by SDS-PAGE and immunoblotted with anti-Ubiquitin antibody (Sigma-Aldrich Canada Co., Oakville, ON) [197].

2.14 Subcellular fractionation

Four different subcellular fractions were prepared using Calbiochem ProteoExtract Subcellular Extraction Kit (Millipore Canada) according to the supplier's protocol [37]. Confluent monolayers of EE2 cells transfected with His-LSP1 and SUMO1; His-LSP1, SUMO1 and SENP1 or His-LSP1^{K270A} and SUMO1 were subjected to four different lysis buffers (I-IV) in a sequential manner. Buffers were supplemented with protease inhibitor cocktails and necessary enzymes. All the fractions (fraction I, cytosol; Fraction II, membrane/organelle; Fraction III, nuclear material and Fraction IV, cytoskeleton) were collected and used for immunoblotting.

2.15 ICAM-1 cross-linking

ICAM-1 cross-linking was used to mimic leukocyte adhesion. To study the effect of ICAM-1 cross-linking *in vivo*, circulating neutrophils were depleted in WT mice by intraperitoneal administration of 200 µg of anti-mouse Ly-6G (Gr-1) antibodies (1 mg/ml; clone RB6-8C5, eBioscience) 24 h prior to treatment with cross-linking antibodies (62), thus ruling out the detection of neutrophil LSP1. For ligation of ICAM-1, rat anti-mouse ICAM-1 antibody (100 µg/mouse; clone YN1/1.7.4, eBioscience) and the respective isotype control (100 µg/mouse rat IgG2b κ; eBioscience) were administered as described previously (43). Saline was administered to control mice. ICAM-1 expression was upregulated by an intrascrotal injection of TNF-α (300 ng) 20 h after administration of Gr-1 antibody. After 4 h of TNF-α treatment, ICAM-1 ligation antibody or isotype control was infused via the jugular vein followed by the secondary anti-rat crosslinking IgG2b (1:100 dilution; clone R2B-7C3, eBioscience). After 30 min of ICAM-1 cross-linking, the cremaster muscle from the TNF-α-treated scrotum was carefully excised and snap-frozen in liquid nitrogen for the detection of total and phosphorylated LSP1 and p38 MAPK by immunoblotting.

ICAM-1 engagement *in vitro* was performed as previously described (50) by incubation of TNF-α-pretreated (20 ng/ml, 4 hours) endothelial cells (EE2) with ICAM-1 ligation antibody or the isotype control antibody for 30 min. Following two washes with pre-warmed (37°C) PBS, cells were incubated with secondary cross-linking antibody for 30 min. Endothelial cells were then prepared for immunoblotting to determine the abundance of phosphorylated LSP1 and total LSP1.

2.16 Functional blocking of β_2-integrins and ICAM-1

For determination of the role of β_2-integrins in KO neutrophil adhesion and endothelial LSP1 phosphorylation, freshly isolated and unstimulated KO bone marrow neutrophils were preincubated with CD18 blocking antibodies (10 µg/ml; clone M18/2, BD Pharmingen) or the isotype control (10 µg/ml rat IgG2a κ; clone R35-95, BD Pharmingen) for 20 min at 37°C, as described elsewhere (38), prior to incubation with MIP-2 (10 min, 37°C). Next, the neutrophils were allowed to adhere to murine heart (HVEC) or lung (LVEC) endothelial cells from WT mice or EE2 cells.

To study the role of ICAM-1, following stimulation with TNF-α (20 ng/ml, 4 h), murine EE2 endothelial cells were treated with ICAM-1 blocking antibody (10 µg/ml; clone YN1/1.7.4,

eBioscience, San Diego, CA) or isotype control (10 μg/ml rat IgG2b κ; eBioscience) for 1 h, as described elsewhere (60). Then, freshly isolated and unstimulated KO bone marrow neutrophils were added on the endothelial cells. After incubation for 30 min, non-adherent neutrophils were removed by two washes with pre-warmed (37°C) PBS. Endothelial cells were lysed for detection of phosphorylated LSP1 or fixed and stained for counting of the number of adherent neutrophils.

2.17 Immunoprecipitation and Nickel-affinity pull-down

His-LSP1-, HA-Ubiquitin- or GFP-moesin-overexpressed HEK 293T or EE2 cells or native EE2 cells were treated as indicated in the figure legends, washed twice with ice-cold PBS and lysed in a lysis buffer containing 150 mM sodium chloride, 1.0% NP-40, 50 mM Tris, pH 8.0 supplemented with protease inhibitor cocktail (Sigma-Aldrich Canada Co., Oakville, ON) on ice. Cell lysates were incubated with appropriate antibody for 2 hours at 4°C in a rotary incubator. Protein A-agarose (Sigma Chemical Co., St. Louis, MO) beads were added to the lysate to catch the respective antibodies. The beads were washed five times with the lysis buffer without protease inhibitor. The immunecomplexes were collected from the beads by adding 50 μl of Laemmli sample buffer and heating at 95°C for 5 min followed by centrifugation in a microfuge for 2 min. Nickel affinity pull-down under native condition was performed at 4°C.

Nickel affinity pull-down under native condition was performed at 4°C according to a protocol described earlier [88]. HEK 293T cells transfected with various expression plasmids and treated as indicated in the figure legends, were lysed in Ni-NTA lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 0.05% Tween, pH 8) containing 10 mM imidazole supplemented with protease inhibitor cocktail (Sigma-Aldrich) on ice. Cell lysates were centrifuged at 10,000×g for 10 min in Eppendorf microcentrifuge tubes to remove cell debris. His-tagged proteins were pulled-down overnight by Ni-NTA agarose slurry (QIAGEN) and washed four times with the lysis buffer containing 10 mM imidazole and once with the same lysis buffer containing 20 mM imidazole. After the last wash, His-tagged proteins were eluted in the lysis buffer containing 250 mM imidazole.

2.18 Immunoblotting

For native protein samples, immunoprecipitation and Nickel-affinity pull-down, cells were lysed in a lysis buffer (pH 8.0) containing 150 mM NaCl, 50 mM Tris, 1% NP-40, and protease and phosphatase (whenever phosphorylated proteins were of interest) inhibitor cocktails (Fisher

Scientific, Toronto, ON, Canada), the lysate was centrifuged (10,000×g, 4°C, 10 min), and the supernatant was collected and stored at -80°C. The same protocol was followed to prepare homogenates from mouse cremaster muscle. For the detection of phosphorylated LSP1, immunoblotting was performed in the non-reduced and non-denatured state, as described previously (8). In brief, the native protein samples were resolved in Mini-PROTEAN TGX precast gels (Bio-Rad, Mississauga, ON, Canada) by electrophoresis. Proteins were transferred onto an Immobilon-FL membrane (Millipore, Billerica, MA) followed by dual blotting of total and phosphorylated LSP1. Total LSP1 was detected using rabbit α-LSP1 serum (1:500 dilution; a generous gift from Dr. J. Jongstra, University of Toronto) and goat α-rabbit IgG tagged with Alexa Fluor 647 (1:5,000 dilution; Invitrogen, Burlington, ON, Canada) as the secondary antibody. Phosphorylated LSP1 was detected using mouse monoclonal antibody against phosphorylated LSP1 (recognizing phosphorylated serine 195 and surrounding residues; 1:500 dilution; clone AT-1E6, Cyclex, Nagano, Japan) and goat α-mouse IgG tagged with Alexa Fluor 488 (1:5,000 dilution; Invitrogen) as the secondary antibody. Membranes were developed with Versa Doc 5000 using appropriate filters. Band densities were quantified using ImageJ. As proteins in their native condition do not run according to their molecular weight, the identity of the protein was confirmed using mass spectrometry in manually excised bands from Coomassie blue stained gel.

All of the other proteins of interest were detected in reduced and denatured conditions, as previously described (29). Protein samples as prepared and mentioned above were solubilized in equal volume of Laemmli sample buffer at 95°C and resolved by 10% SDS-PAGE. For immunoblotting, proteins were transferred onto a nitrocellulose membrane and blocked with 5% BSA in Tris-buffered saline-Tween 20 at room temperature for 1 h. Then the membrane was incubated with affinity-purified rabbit-α-PECAM-1 (1:1000 dilution; Thermo Fisher Scientific), rat-α-ICAM-1 (1: 1000 dilution; eBioscience), rat-α-VCAM-1 (1:1000 dilution; Abcam Inc, Toronto, ON, Canada), rabbit-α-GATA-2 (1:1000 dilution; Santa Cruz Biotechnology), α-LSP1 rabbit serum (1:1000 dilution; a generous gift from Dr. J. Jongstra, University of Toronto), mouse-α-GFP, rabbit-α-His, mouse-α-moesin, rabbit α-phosphorylated p38 MAPK (1:1,000 dilution; Cell Signaling Technology, Danvers, MA), rabbit α-p38 antibody (1:1,000 dilution; Cell Signaling Technology), rabbit-α-phosphorylated moesin (recognizing phosphorylated threonine 558 and surrounding residues; 1:500 dilution; Santa Cruz Biotechnology) or mouse-α-β-actin (1:2500 dilution; Santa Cruz Biotechnology) antibody at 4°C overnight. After incubation with horseradish

peroxidase-conjugated respective secondary antibody (1:5000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature, antibody binding was detected with enhanced chemiluminescence detection reagent (GE Healthcare, Baie d'Urfe, QC, Canada). ImageJ software (v1.47; http://rsbweb.nih.gov/ij) was used for densitometric quantification of the detected bands. Intensity values for the proteins were normalized to β-actin.

2.19 Mass spectrometry

Polyacrylamide gel bands containing protein of interest were excised and processed using a MassPrep Station (Waters, Milford, MA, USA) using the methods supplied by the manufacturer [198]. Briefly, the gel fragments were first destained, reduced, alkylated, digested with trypsin, and extracted overnight at room temperature. The resulting tryptic digest was then analyzed by mass spectrometry. For electrospray, quadruple time-of-flight (Q-TOF) analysis, 1 µl of the solution was used. Liquid chromatography/mass spectrometry (LC/MS) was performed on a CapLC high-performance liquid chromatography unit (Waters) coupled with Q-TOF-2 mass spectrometer (Micromass). A mass deviation of 0.2 was tolerated and one missed cleavage site was allowed. Resulting values from mass spectrometry analysis (MS/MS) were used to search against the SwissProt database with Rodentia specified. We used the Mascot (www.matrixscience.com) search engine to search the protein database.

2.20 RT-qPCR

RT-PCR was performed to determine PECAM-1, ICAM-1 and β-actin mRNA expression in EE2 cells or LSP1 and β-actin mRNA expression in His-LSP1 or His-LSP1^{K270A} transfected HEK 293T cells as described previously [199]. Briefly, total RNA was extracted from respective cells using the RNEasy Mini Kit (Qiagen, Toronto, ON) and reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Toronto, ON). Relative levels of specific mRNAs were determined with the QuantiTect SYBR Green PCR Kit (Qiagen, Toronto, ON) and predesigned primers targeting murine PECAM-1 (QT01052044; Qiagen), ICAM-1 (QT00155078; Qiagen), β-actin (QT00095242; Qiagen), LSP1 (QT01046227; Qiagen, Toronto, ON) and human β-actin (QT00095431; Qiagen, Toronto, ON). All PCRs were performed in triplicate and run for 30 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec.

2.21 Confocal imaging

EE2 cells were cultured on glass coverslips, co-transfected with GFP-LSP1 and pCMV-SPORT6-SUMO1 plasmid, and allowed to grow for 24 hours. Medium was removed and the cells were washed once with 37°C pre-warmed PBS before they were fixed in 4% formaldehyde for 30 min, washed thrice with PBS, permeabilized with 0.1% Triton X-100 and blocked with 5% BSA for 30 min. The cells were incubated with mouse anti-SUMO1 monoclonal antibody (Life Technologies Inc., Burlington, ON) for 2 hours at room temperature, washed thrice in PBS and incubated with Alexa Fluor 532 conjugated goat anti-mouse IgG (Life Technologies Inc., Burlington, ON) in the dark at room temperature for 1 hour. Coverslips were mounted on glass slides using prolong gold anti-fade reagent (Life Technologies Inc., Burlington, ON), the edges of the coverslips were sealed with a nail polish and visualized with a laser scanning confocal microscope (Zeiss, ConfoCor2/LSM510) [199].

2.22 Statistical analysis

Data are shown as arithmetic means ± SD or SEM. Statistical analysis was made using Student *t* test or ANOVA with Tukey's post-hoc comparison test. *n* denotes the number of different mice, different batches of endothelial cells or neutrophils studied in each group. Values of $P < 0.05$ were considered statistically significant.

3.0 ICAM-1-MEDIATED NEUTROPHIL ADHESION IS CRITICAL FOR LEUKOCYTE-SPECIFIC PROTEIN 1 (LSP1) PHOSPHORYLATION

A major part of the data presented in this chapter were used in a research paper entitled "ICAM-1-mediated leukocyte adhesion is critical for the activation of endothelial LSP1" published by Mokarram Hossain, Syed M. Qadri, Yang Su and Lixin Liu in the *American Journal of Physiology – Cell Physiology*, 2013; 304: C895–C904.

3.0 ICAM-1-MEDIATED NEUTROPHIL ADHESION IS CRITICAL FOR LEUKOCYTE-SPECIFIC PROTEIN 1 (LSP1) PHOSPHORYLATION

In acute inflammation, leukocyte adhesion on the endothelium is mediated by the β_2-integrins, e.g., LFA-1 and Mac-1 [13, 200-202]. Chemokine stimulation upregulates surface expression of integrins on leukocytes which allows enhanced binding of leukocyte integrins to endothelial ligands such as ICAM-1 [203, 204]. This binding, in turn, initiates ICAM-1 signaling in endothelial cells which is mediated by receptor multimerization [205] and modulates the actin cytoskeleton by interacting with ERM proteins (ezrin, radixin, moesin), β-tubulins, caveolin-1, cortactins, and filamins A and B [15, 22, 26, 27, 206]. ICAM-1 engagement also triggers tyrosine phosphorylation of cytoskeleton-associated proteins, such as focal adhesion kinase, paxillin, and p130cas [28], and phosphorylation of junctional proteins, such as vascular endothelial cadherin, which fosters the subsequent transendothelial migration of leukocytes [29, 30]. Leukocyte adhesion-triggered ICAM-1 signaling regulates transcellular and paracellular leukocyte transmigration [31] and microvascular permeability increases [207].

Despite being biochemically almost identical, functional differences between neutrophil and endothelial LSP1 are remarkable. In neutrophils, LSP1 is mainly distributed in the cytoplasm [138] and serves as a substrate for PKC and p38 MAPK [143, 152]. Contradictory reports are available concerning regulatory roles of neutrophil LSP1 on adhesion, polarization, and migration [147]. However, in endothelial cells, LSP1 is mostly located in the nuclei, with small level of LSP1 found in F-actin-rich microfilaments [136]. Interestingly, LSP1-deficient (KO) mice showed a compromised formation of endothelial transmigratory dome-like structures, resulting in a disproportionate increase in vascular permeability, despite impaired transendothelial migration of leukocytes [32, 37]. In leukocytes, LSP1 has been shown to be activated by chemoattractants [208]. However, the mechanisms of endothelial LSP1 activation during leukocyte recruitment remain completely unknown.

3.1 Activation of either neutrophils or endothelial cells results in similar level of neutrophil adhesion *in vitro*

In neutrophils, functions of LSP1 are thought to be regulated by the phosphorylation of LSP1 by MAPK-activated protein kinase 2 (MK2), a specific downstream target of p38 MAPK whereas in T cells, protein kinase C-mediated LSP1 phosphorylation is more

important [143, 153, 182]. Phosphorylated LSP1 was shown to be colocalized with F-actin at the leading edge of the fMLP-induced polarized neutrophils [208]. Moreover, phosphorylation of LSP1 was documented to be the link between p38 MAPK activation and cytoskeletal reorganization in bacterial homoserine lactone AHL-12-induced chemotaxing neutrophils [34]. In addition, in dendritic cells, using pharmacological inhibitors, it was shown that viral protein gp120-induced dendritic cell migration and phosphorylation of LSP1 was p38 MAPK-dependent [35]. In hematopoietic cells, phosphorylation was shown to be essential for the activation of LSP1 which can be induced by numerous types of stimulants, e.g., chemokine, bacterial peptide or viral protein. However, phosphorylation of endothelial LSP1 has not been reported and it is yet to be determined whether similar stimuli can phosphorylate LSP1. It is well known that binding of surface adhesion molecules of leukocytes with their ligands on endothelium itself can trigger a large number of endothelial signaling events [28, 36, 38, 45, 205]. To address this issue, we investigated whether chemokine, cytokine or neutrophil adhesion stimulates phosphorylation of endothelial LSP1. Activation of endothelial cells with TNF-α is a common event in inflammation. However, TNF-α-treatment itself initiates multiple signaling in endothelial cells including p38 MAPK and Rho kinase activation. To avoid this, we first established an *in vitro* adhesion assay where activated neutrophils were allowed to adhere onto unstimulated endothelial monolayers. In this connection, we stimulated neutrophils with chemokines (KC and MIP-2) and a calcium signal-initiation activator called thapsigargin (designated as TG). Using a series of thapsigargin concentration, we found 100 nM to be the most effective thapsigargin dose for activating neutrophil (Figure 3.1A). We then stimulated neutrophils isolated from KO mice with TG, KC and MIP-2 and allowed them to adhere onto unstimulated endothelial cells for 30, 60 or 90 min just after washing once with PBS. Neutrophil adhesion was significantly higher in all the treatment groups compared to control at all time points. For the subsequent studies, we used 30 min incubation time for adhesion assays since it was sufficient to show the difference and at this time point adhesion was at a very similar level in all the treatment groups (Figure 3.1B). Finally, we compared whether adhesion level is similar when endothelial cells are activated by TNF-α treatment (20 ng/ml; 4 hours). As illustrated in Figure 3.1C, stimulation of neutrophils with MIP-2 or thapsigargin or of endothelial cells with TNF-α significantly enhanced the number of adherent neutrophils. Figure 3.1C also shows that activation of either neutrophil or endothelial cells provided similar level of neutrophil adhesion onto EE2 cell monolayers.

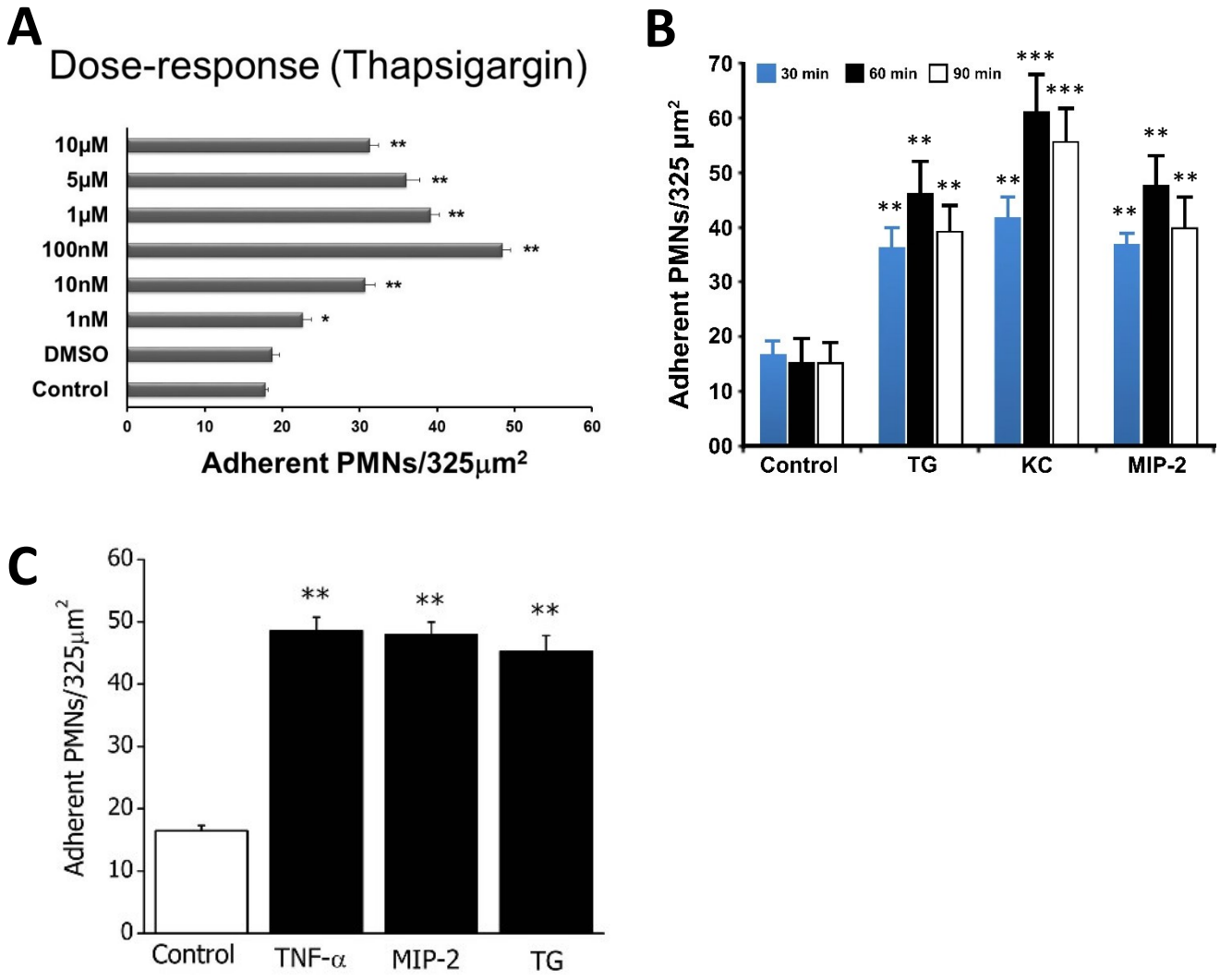

Figure 3.1: Activation of either endothelial cells or neutrophils is sufficient to ensure neutrophil adhesion onto endothelial monolayers. **(A)** Number of adherent KO neutrophils (counted in a 325-μm^2 area) in the absence (control) and presence of stimulation with a wide range of thapsigargin doses or DMSO was determined after 30 min of incubation on EE2 cells. Values are means ± SD (n = 4). **Significantly different (P < 0.01) from control. **(B)** Number of adherent KO neutrophils (counted in a 325-μm^2 area) in the absence (control) and presence of stimulation with 100 nM thapsigargin (TG), 100 nM keratinocyte chemokine (KC) or 100 nM macrophage inflammatory protein-2 (MIP-2) was determined after 30, 60 or 90 min of incubation on EE2 cells. Values are means ± SD (n = 3). **; ***Significantly different (P < 0.01; < 0.001) from control. **(C)** Number of adherent KO neutrophils (counted in a 325-μm^2 area) on murine EE2 endothelial cells in the absence (control) and presence of stimulation with 100 nM MIP-2 or after pretreatment of neutrophils with 100 nM thapsigargin (TG). Unstimulated neutrophils were allowed to adhere to endothelial cells stimulated with 20 ng/ml TNF-α. Values are means ± SD (n = 3–6). **Significantly different (P < 0.01) from control.

3.2 Neutrophil adhesion is critical for endothelial LSP1 phosphorylation

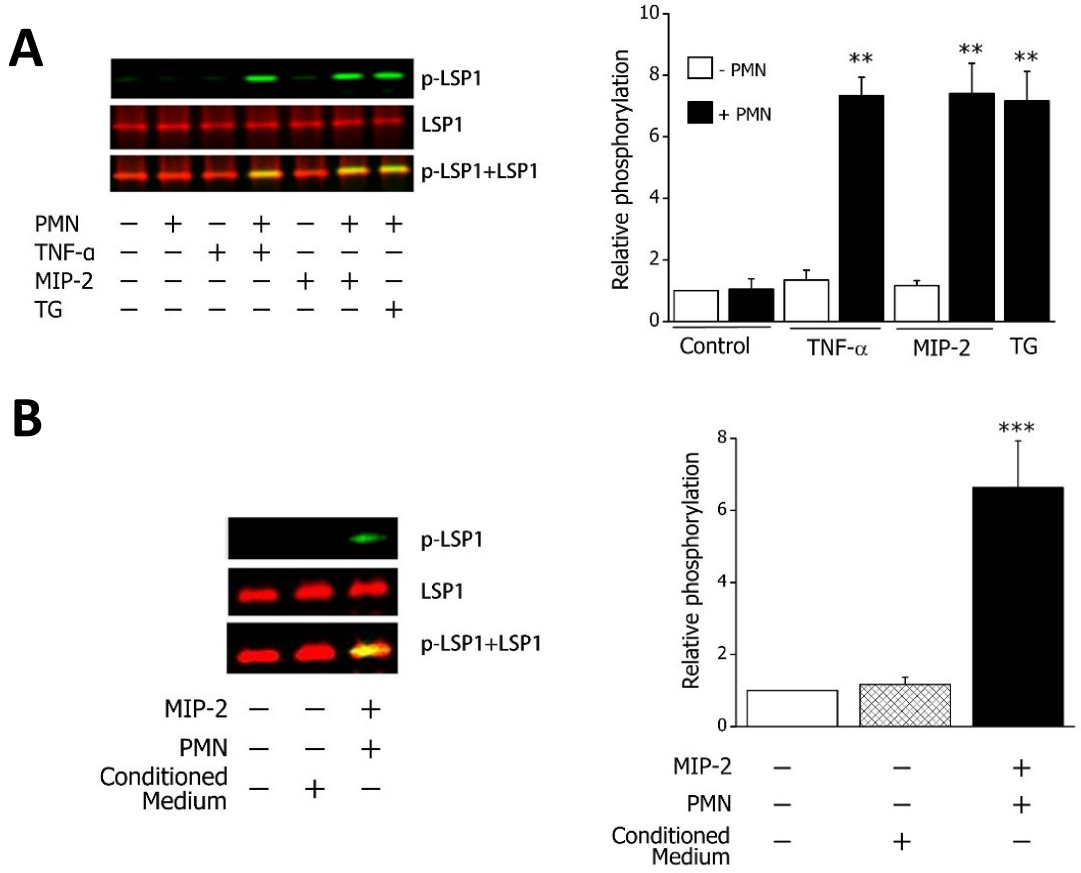

Figure 3.2: Phosphorylation of endothelial LSP1 by neutrophil (PMN) adhesion. (A) *Left panel:* original dual immunoblots (n = 3) demonstrating expression of phosphorylated LSP1 (p-LSP1, green), total LSP1 (LSP1, red), and overlay (p-LSP1 + LSP1, yellow) in endothelial cells after 30 min in the presence (+) or absence (−) of neutrophils with (+) or without (−) stimulation with 20 ng/ml TNF-α or 100 nM MIP-2 or pretreatment with 100 nM thapsigargin. *Right panel:* densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 in endothelial cells after 30 min in the presence or absence of neutrophils with or without stimulation with 20 ng/ml TNF-α or 100 nM MIP-2 or pretreatment with 100 nM thapsigargin. Values are means ± SD (n = 3). **Significantly different ($P < 0.01$) from −PMN with TNF-α or MIP-2 or +PMN (by ANOVA). **(B)** *Left panel:* original dual immunoblots (n = 3) demonstrating expression of phosphorylated LSP1, total LSP1, and overlay in endothelial cells after 30 min of incubation in the presence or absence of neutrophils, MIP-2 (100 nM), or conditioned medium collected from MIP-2-elicited neutrophil adhesion to endothelial cells. *Right panel:* densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 in endothelial cells after 30 min of incubation with or without neutrophils, MIP-2 (100 nM), or conditioned medium collected from MIP-2-elicited neutrophil adhesion to endothelial cells. Values are means ± SD (n = 3). ***Significantly different ($P < 0.001$) from controls without neutrophils or with conditioned medium collected from MIP-2-elicited neutrophil adhesion to endothelial cells (by ANOVA).

In the adhesion assay, we used KO neutrophils to rule out the contribution of leukocyte-expressed LSP1 in the detection of phosphorylated LSP1 after the adhesion assay. Phosphorylated LSP1 was quantified in endothelial cells in the presence and absence of adherent KO neutrophils. Neutrophils stimulated with MIP-2 or pretreated with thapsigargin and allowed to adhere onto endothelial cells significantly increased phosphorylated LSP1 in endothelial cells compared with endothelial cells treated with MIP-2 alone or with untreated neutrophils (Figure 3.2A). Similarly, the addition of untreated KO neutrophils to TNF-α-stimulated endothelial cells significantly augmented endothelial LSP1 phosphorylation. In contrast, TNF-α stimulation did not enhance phosphorylation of endothelial LSP1 in the absence of neutrophils. These data clearly indicate that adhesion of neutrophils to endothelial cells is critical for LSP1 phosphorylation in endothelial cells which is not affected by cytokine or chemokine stimulation alone (Figure 3.2A).

During adhesion, neutrophils may secrete a variety of soluble mediators and substances that might lead to activation of endothelial LSP1. To exclude this possibility, we tested whether the conditioned medium harvested after neutrophil adhesion to endothelial cells triggered the activation of endothelial LSP1. As shown in Figure 3.2B, not the presence of conditioned medium and MIP-2 but, rather, the adherence of KO neutrophils in the presence of MIP-2 elicited phosphorylation of endothelial LSP1. Mass spectroscopic analysis of the protein bands confirmed the identity of mouse endothelial LSP1 (Mowse/ions score: 881; queries matched: 42; sequence coverage: 52%; pI/mol wt: 4.7/36.7). Ion score is $-10\times\log(P)$, where P is the probability that the match is a random event. Individual ion score >32 indicates identity or extensive homology ($P < 0.05$).

3.3 p38 MAPK plays a major role in endothelial LSP1 phosphorylation

In neutrophils, activation of LSP1 is effectively accomplished by the phosphorylation of LSP1 in Ser243 by p38 MAPK-mediated signaling events downstream of receptor-mediated activation signals from chemoattractants [34, 143, 208]. We provided direct and specific experimental verification that phosphorylation of endothelial LSP1 is blunted by pharmacological inhibition of p38 MAPK. It is documented that inhibition of p38 MAPK signaling downregulates the expression of β_2-integrins [209] and decreases leukocyte adhesion to endothelial cells [10, 40-42]. In light of these results, LSP1 phosphorylation in endothelial cells could be diminished as a result of decreased neutrophil adhesion to endothelial cells. To address this issue, we analyzed neutrophil adhesion using different ratios of added KO neutrophils to endothelial cells, which

affected the number of adherent cells. As the first step, we show that neutrophil adhesion and abundance of phosphorylated endothelial LSP1 were significantly enhanced with increased neutrophil-to-endothelial cell ratio (Figure 3.3A–C). As illustrated in Figure 3.3D, we exploited the effects of increased neutrophil-to-endothelial cell ratio to demonstrate that the number of adherent neutrophils was significantly higher at 2:1 than 1:1 neutrophil-to-endothelial cell ratio, and addition of SB-203580 (10 μM) significantly decreased the number of adherent neutrophils. However, the number of adherent neutrophils in the group with a 1:1 neutrophil to endothelial cell ratio and without SB-203580 did not significantly differ from the neutrophil adhesion number in the group with a 2:1 neutrophil-to-endothelial cell ratio and in the presence of SB-203580 (Figure 3.3D). Accordingly, analysis of phosphorylated LSP1 in these treatment groups showed that pharmacological inhibition of p38 MAPK by SB-203580 indeed blunted the phosphorylation of endothelial LSP1, despite similar numbers of adherent neutrophils (Figure 3.3D–F). This indicates that phosphorylation of endothelial LSP1 is downstream of p38 MAPK signaling in endothelial cells.

In neutrophils and T cells, LSP1 was previously described as a substrate for PKC [153, 182, 208]. A further series of experiments were performed to elucidate the role of PKC in the modulation of endothelial LSP1 phosphorylation triggered by neutrophil adhesion. Treatment of endothelial cells with the PKC inhibitors staurosporine (100 nM), sotrastaurin (5 μM), or Gö-6983 (1 μM) tended to decrease neutrophil adhesion triggered by MIP-2, an effect not reaching statistical significance. The number of adherent neutrophils (counted in a 325-μm^2 area) was 38.3 ± 7.3, 40.2 ± 6.5, and 41.2 ± 5.9 in the presence of staurosporine, sotrastaurin, and Gö-6983, respectively, compared with 49.2 ± 3.7 in the control group without PKC inhibition (n = 3 each, P >0.05, ANOVA). Similarly, enhanced expression of phosphorylated endothelial LSP1 triggered by MIP-2-activated neutrophils did not significantly differ upon treatment with staurosporine, sotrastaurin, or Gö-6983. The relative LSP1 phosphorylation was 65.0 ± 7.0 in the absence of PKC inhibitors and 47.3 ± 10.2, 49.6 ± 9.9, and 49.8 ± 8.5 in the presence of staurosporine, sotrastaurin, and Gö-6983 (arbitrary units, n = 3 each, P > 0.05, ANOVA), respectively. Clearly, p38 MAPK plays a more prominent role than PKC in endothelial LSP1 activation.

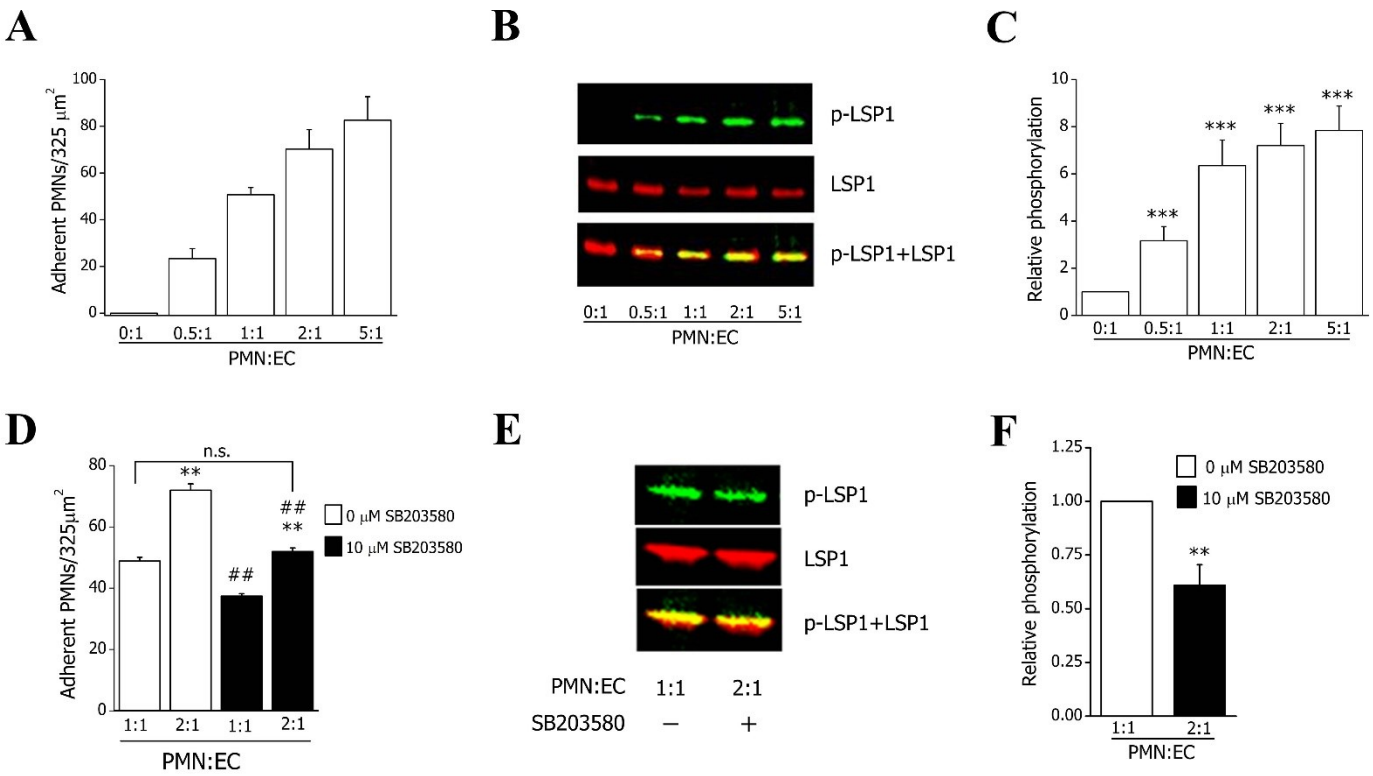

Figure 3.3: Suppression of endothelial LSP1 phosphorylation by pharmacological inhibition of p38 MAPK. **(A)** Number of adherent KO neutrophils (counted in a 325-μm2 area) stimulated with MIP-2 (100 nM) on murine EE2 endothelial cells at neutrophil-to-endothelial cell ratio (PMN:EC) of 0:1, 0.5:1, 1:1, 2:1, and 5:1. Values are means ± SD (n = 3). **(B)** Original dual immunoblots (representative of 3 similar experiments) demonstrating expression of phosphorylated LSP1 (Alexa Fluor 488, green), total LSP1 (Alexa Fluor 647, red), and overlap of phosphorylated and total LSP1 (yellow) in endothelial cells with neutrophil-to-endothelial cell ratios of 0:1, 0.5:1, 1:1, 2:1, and 5:1. **(C)** Densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 in endothelial cells with neutrophil-to-endothelial cell ratios of 0:1, 0.5:1, 1:1, 2:1, and 5:1. Values are means ± SD (n = 3). ***Significantly different ($P < 0.001$) from 0:1 (by ANOVA). **(D)** Number of adherent KO neutrophils (counted in a 325-μm2 area) stimulated with MIP-2 (100 nM) on primary endothelial cells from WT mice in neutrophil-to-endothelial cell ratios of 1:1 and 2:1, in the absence (0 μM) and presence of the specific p38 MAPK inhibitor SB-203580 (10 μM). Values are means ± SD (n = 3). **Significantly different ($P < 0.01$) from 1:1 (by ANOVA). ##Significantly different ($P < 0.01$) from 0 μM SB-203580 (by ANOVA). ns, Not significant. **(E)** Original dual immunoblots (representative of 3 similar experiments) demonstrating expression of phosphorylated LSP1 (Alexa Fluor 488, green), total LSP1 (Alexa Fluor 647, red), and overlap of phosphorylated and total LSP1 (yellow) in the absence and presence of SB-203580 (10 μM) with neutrophil-to-endothelial cell ratios of 1:1 and 2:1. **(F)** Densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 of endothelial cells in the absence and presence of SB-203580 (10 μM) with neutrophil-to-endothelial cell ratios of 1:1 and 2:1. Values are means ± SD (n = 3). **Significantly different ($P < 0.01$) from 0 μM SB-203580 (by Student's t-test).

3.4 β_2 integrins and ICAM-1 are involved in neutrophil adhesion and subsequent endothelial LSP1 phosphorylation

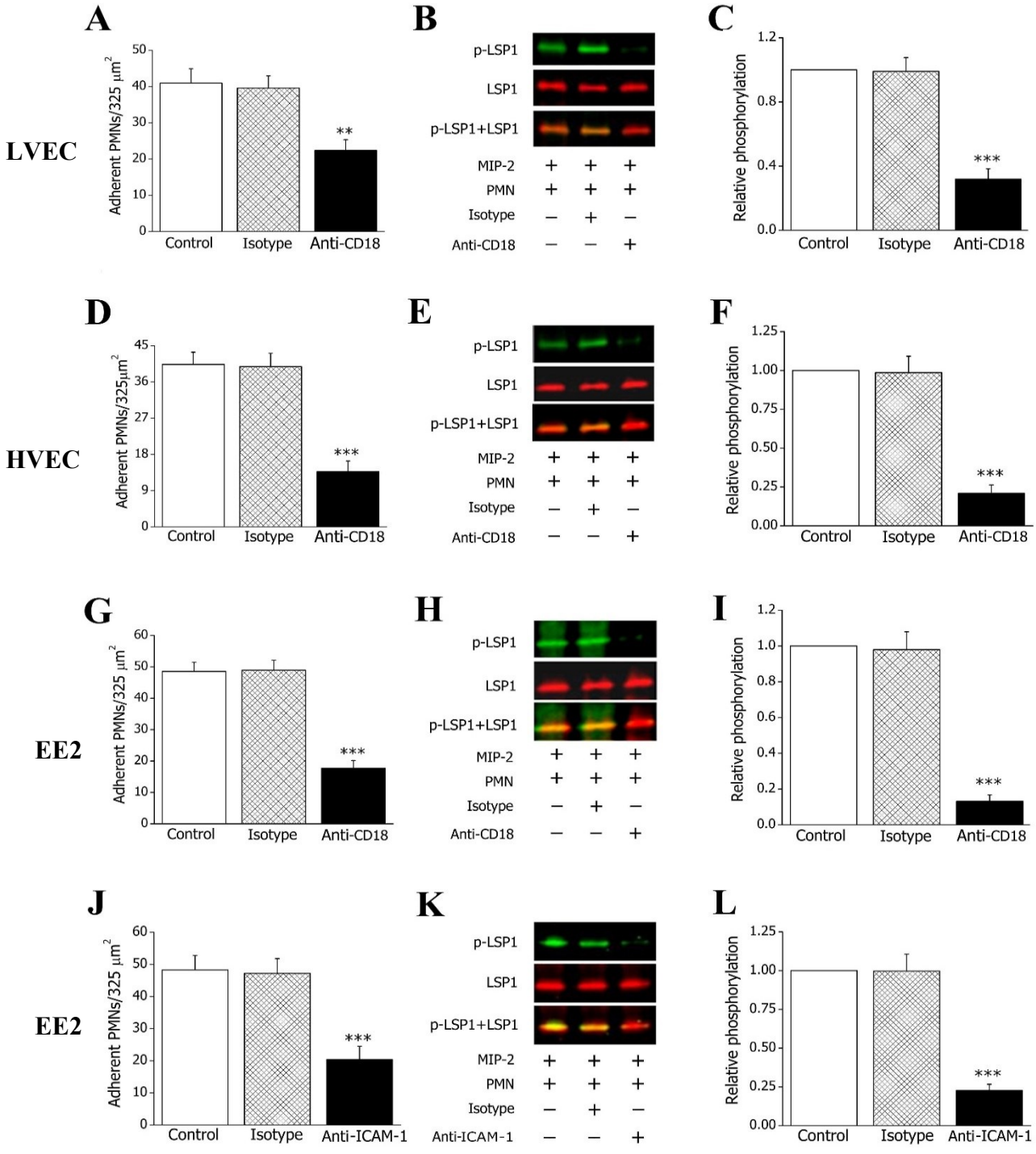

Figure 3.4: Blocking neutrophil β_2-integrins/CD18 or endothelial ICAM-1 inhibits neutrophil adhesion and phosphorylation of endothelial LSP1. **(A, D,** and **G)** number of adherent KO neutrophils (counted in a 325-μm^2 area) stimulated with 100 nM MIP-2 in the absence (control)

and presence of β_2-integrin blocking antibodies or respective isotype control (isotype) on primary lung (LVEC; **A**) and heart (HVEC; **D**) endothelial cells from WT mice and murine EE2 endothelial cells **(G)**. Values are means ± SD (n = 3). Significantly different from control and isotype: $^{**}P < 0.01$ and $^{***}P < 0.001$ (by ANOVA). **(B, E,** and **H)** original dual immunoblots (n = 3) demonstrating expression of phosphorylated LSP1 (green), total LSP1 (red), and overlay of phosphorylated and total LSP1 (yellow) in primary lung **(B)** and heart **(E)** endothelial cells from WT mice and murine EE2 endothelial cells **(H)** after 30 min of incubation in the presence of neutrophils stimulated with MIP-2 (100 nM) and in the presence or absence of anti-β_2-integrin blocking antibodies or the respective isotype control antibodies. **(C, F,** and **I)** densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 in primary lung **(C)** and heart **(F)** endothelial cells from WT mice and murine EE2 endothelial cells **(I)** after 30 min of incubation in the presence of neutrophils stimulated with MIP-2 (100 nM) and in the absence (control) or presence of anti-β_2-integrin blocking antibodies or the respective isotype control antibodies. Values are means ± SD (n = 3). ***Significantly different ($P < 0.001$) from control and isotype (by ANOVA). **(J)** Number of adherent KO neutrophils (counted in a 325-μm^2 area) in the absence (control) and presence of anti-ICAM-1 blocking antibodies or the respective isotype control on TNF-α-stimulated (20 ng/ml, 4 h) murine EE2 endothelial cells. Values are means ± SD (n = 3). ***Significantly different ($P < 0.001$) from control and isotype (by ANOVA). **(K)** original dual immunoblots (representative of 3 similar experiments) demonstrating expression of phosphorylated LSP1 (Alexa Fluor 488, green), total LSP1 (Alexa Fluor 647, red), and overlap of phosphorylated LSP1 and total LSP1 (yellow) determined in TNF-α-stimulated (20 ng/ml, 4 h) murine EE2 endothelial cells after 30 min of incubation in the presence of adherent neutrophils and in the presence or absence of anti-ICAM-1 blocking antibodies or the respective isotype control antibodies. **(L)** densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 in TNF-α-stimulated (20 ng/ml, 4 h) murine EE2 endothelial cells after 30 min in the presence of adherent neutrophils and in the absence (control) or presence of anti-ICAM-1 blocking antibodies or the respective isotype control antibodies. Values are means ± SD (n = 3). ***Significantly different ($P < 0.001$) from control and isotype (by ANOVA).

It has been documented that neutrophil recruitment in the lung can be integrin- and ICAM-1-independent [43, 46]. To test whether these organ-specific mechanisms affect phosphorylation of endothelial LSP1, a series of experiments were performed using blocking antibodies against β_2-integrins to analyze neutrophil adhesion and subsequent LSP1 phosphorylation in endothelial cells derived from different organs. In murine primary lung endothelial cells (LVEC), MIP-2-triggered neutrophil adhesion and subsequent endothelial LSP1 phosphorylation were significantly blunted by the β_2-integrin blocking antibodies (Figure 3.4A–C). Similarly, murine primary heart endothelial cells (HVEC) and EE2 cells showed inhibition of neutrophil adhesion (Figure 3.4D and G) and phosphorylation of endothelial LSP1 (Figure 3.4E, F, H, and I). As depicted in Figure

3.4, blocking neutrophil β_2-integrins resulted in 45%, 65%, and 63% decreased neutrophil adhesion to primary lung endothelial cells, primary heart endothelial cells, and murine EE2 endothelial cells, respectively. Similarly, blocking neutrophil β_2-integrins resulted in 69%, 73%, and 85% reduction of phosphorylated LSP1 in primary lung endothelial cells, primary heart endothelial cells, and EE2 cells, respectively.

To further substantiate our findings, we assessed the effects of blocking endothelial ICAM-1 on neutrophil adhesion and activation of endothelial LSP1 in vitro. Treatment with anti-ICAM-1 blocking antibodies significantly reduced neutrophil adhesion (Figure 3.4J) and resulted in significant inhibition of LSP1 phosphorylation in TNF-α-treated endothelial cells (Figure 3.4K and L). Treatment with isotype control antibodies, however, did not significantly modify neutrophil adhesion to endothelial cells (Figure 3.4J) and the phosphorylation of endothelial LSP1 (Figure 3.4K and L).

3.5 ICAM-1 cross-linking mimics the function of neutrophil adhesion

ICAM-1 is the most important endothelial adhesion molecule for firm neutrophil adhesion. In fact, ICAM-1 cross-linking is routinely used to mimic neutrophil adhesion *in vivo* and *in vitro* [207]. ICAM-1 has been identified as a critical signaling molecule connecting leukocyte adhesion with downstream events in endothelial cells. ICAM-1 cross-linking was previously shown to activate endothelial p38 MAPK signaling [47], which is partially mediated by xanthine oxidase [49]. ICAM-1 engagement by cross-linking antibodies or human rhinovirus binding was also shown to induce phosphorylation of p38 MAPK in a Syk tyrosine kinase-dependent manner [50]. Therefore, to verify that the phosphorylation of endothelial LSP1 induced by KO neutrophils was indeed triggered by the binding through endothelial adhesion molecules, we used ICAM-1 antibody cross-linking, which engages ICAM-1 on endothelial cells, to determine the effect of ICAM-1-mediated adhesion in triggering of endothelial LSP1 phosphorylation. After stimulation of endothelial cells with TNF-α *in vitro*, ICAM-1 cross-linking significantly enhanced the phosphorylation of LSP1 in endothelial cells compared with that in the presence of the control isotype immunoglobulins on TNF-α-treated endothelial cells or the same cross-linking on unstimulated endothelial cells (Figure 3.5). After ICAM-1 ligation and then cross-linking on unstimulated endothelial cells, we observed a subtle, but significant, increase in the abundance of

phosphorylated endothelial LSP1 underlining baseline levels of ICAM-1 expression and its functions in LSP1 activation upon crosslinking on unstimulated endothelial cells (Figure 3.5).

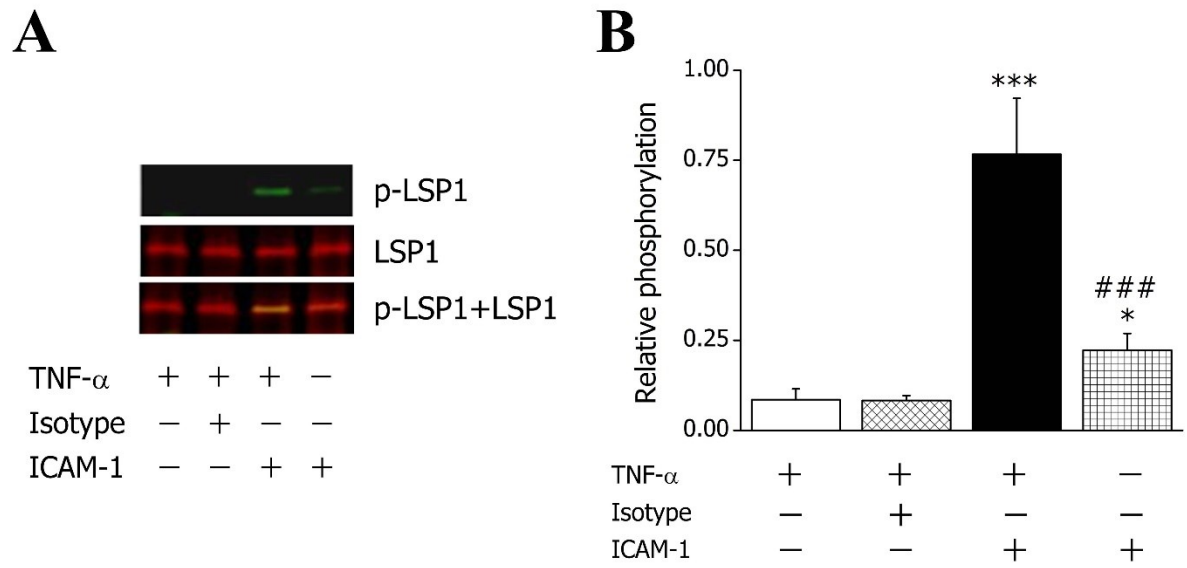

Figure 3.5: Phosphorylation of endothelial LSP1 by ICAM-1 engagement in vitro. **(A)** Original dual immunoblots (n = 3) demonstrating expression of phosphorylated LSP1 (green), total LSP1 (red), and overlay (yellow) determined in endothelial cells after stimulation with TNF-α (20 ng/ml) in the presence of cross-linking antibodies following addition of anti-ICAM-1 antibodies (ICAM-1) or isotype controls (isotype). **(B)** Densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 of endothelial cells determined in endothelial cells without stimulation with TNF-α and in the presence of cross-linking antibodies and after stimulation with TNF-α (20 ng/ml) in the absence (control) or presence of cross-linking antibodies following addition of anti-ICAM-1 antibodies (ICAM-1) or the isotype control. Values are means ± SD (n = 3). Significantly different from control and isotype: *$P < 0.05$ and ***$P < 0.001$ (by ANOVA). ###Significantly different ($P < 0.001$) from cross-linking antibodies + TNF-α stimulation (by ANOVA).

As an additional approach, we determined the effect of ICAM-1 engagement on endothelial LSP1 phosphorylation in vivo. Administration of ICAM-1 cross-linking antibodies to KO mice following depletion of circulating neutrophils significantly enhanced the abundance of phosphorylated LSP1 in cremaster muscle. Administration of isotype control immunoglobulins, however, did not modify the level of phosphorylated LSP1 (Figure 3.6A and B). These results indicate that ICAM-1-mediated adhesion is important for the phosphorylation of endothelial LSP1. Consistent with our findings that pharmacological inhibition of p38 MAPK attenuates phosphorylation of endothelial LSP1 (Figure 3.3), we observed robust phosphorylation of p38

MAPK in cremaster muscle upon ICAM-1 cross-linking, but not upon administration of the isotype immunoglobulins (Figure 3.6C and D).

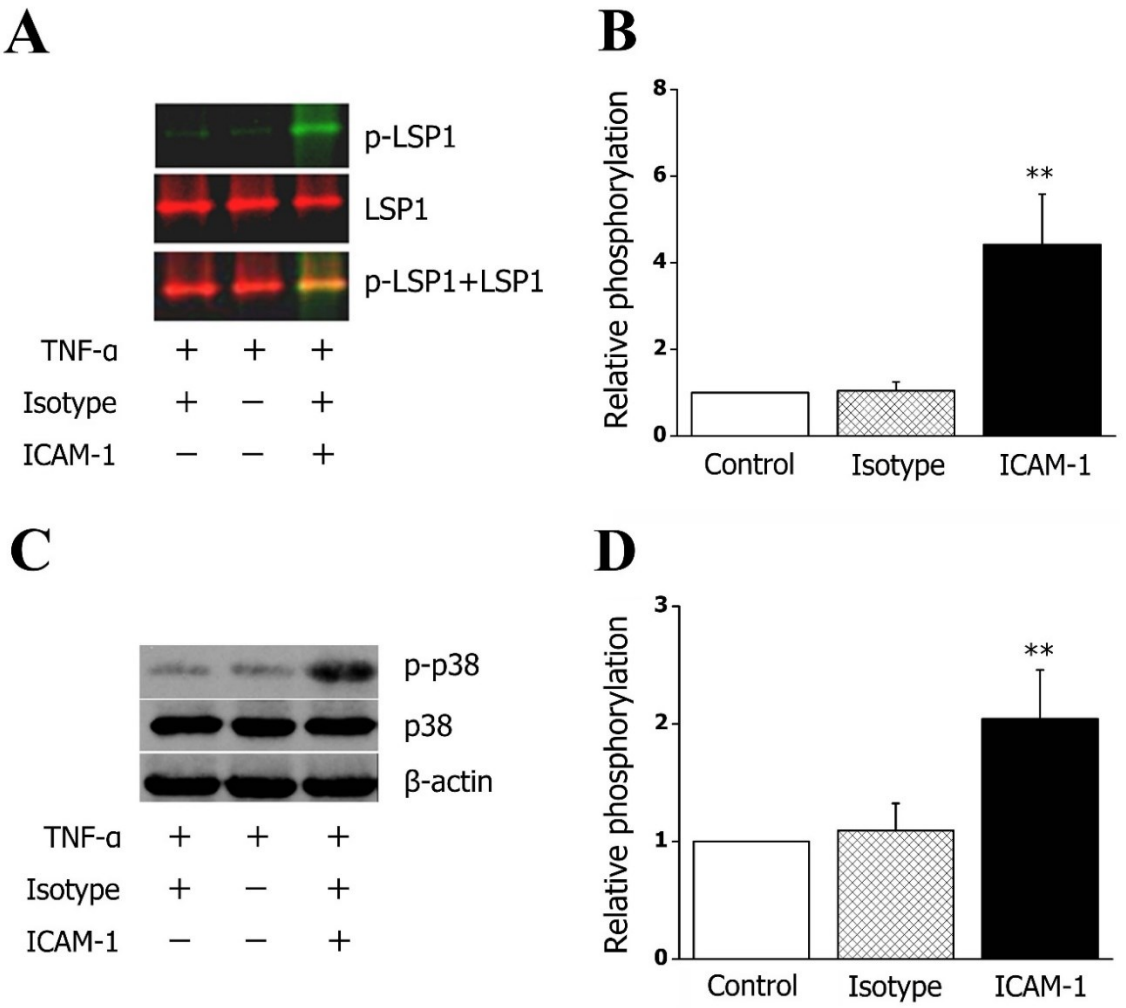

Figure 3.6: LSP1 and p38 MAPK phosphorylation in cremaster muscle by ICAM-1 engagement in vivo. **(A and C)** original dual immunoblots (n = 3) demonstrating expression of phosphorylated LSP1 (green), total LSP1 (red), and overlay (yellow) and expression of phosphorylated p38 MAPK (p-p38), total p38 MAPK (p38), and respective β-actin protein abundance (β-actin) determined in TNF-α-stimulated (300 ng, intrascrotal injection, 4 h) cremaster muscle of neutrophil-depleted WT mice after administration of saline (control) or cross-linking antibodies following addition of anti-ICAM-1 antibodies (ICAM-1) or isotype controls (isotype). **(B** and **D*)*** densitometric analysis of abundance of phosphorylated LSP1 relative to total LSP1 and phosphorylated p38 MAPK relative to total p38 MAPK determined in TNF-α-stimulated (300 ng, intrascrotal injection, 4 h) cremaster muscle of neutrophil-depleted WT mice after administration of saline (control) or cross-linking antibodies following addition of anti-ICAM-1 antibody (ICAM-1) or isotype controls. Values are means ± SD (n = 3). **Significantly different ($P < 0.01$) from control or isotype (by ANOVA).

Interestingly, leukocyte adhesion further enhanced the phosphorylation of endothelial p38 MAPK elicited by TNF-α, pointing to a synergism between leukocyte adhesion and cytokine stimulation [17]. Our observation that basal abundance of phosphorylated p38 MAPK in TNF-α-stimulated cremaster muscle tissue was increased after ICAM-1 crosslinking is consistent with a previous observation [210]. Moreover, various other studies have shown that ICAM-1 engagement may also induce PKC activation [18, 23, 211]. It is intriguing to speculate that endothelial LSP1 phosphorylation following leukocyte adhesion contributes to the physiological functions of microvascular permeability and transendothelial migration of leukocytes, thus explaining the impaired inflammatory phenotypes in KO mice [32, 37, 136].

3.6 Summary

This is the first report on the phosphorylation of endothelial LSP1. Here, we demonstrate that the adhesion of neutrophils to endothelial cells but not the stimulation with chemokine or cytokine alone, triggers phosphorylation of endothelial LSP1. This phosphorylation was easily, though partially, inhibited by inhibiting adhesion via functional blocking of neutrophil-expressed β_2 integrins or endothelial cell-expressed ICAM-1, the most important adhesion molecules involved in firm neutrophil adhesion to endothelial cells. The role of ICAM-1 as the initiator of this signaling event was established by *in vitro* and *in vivo* ICAM-1 cross-linking studies. ICAM-1 engagement is known to activate p38 MAPK and PKC. Both of them are capable of phosphorylating LSP1 in other cell types. However, using pharmacological inhibitor of both p38 MAPK and PKC, we could establish the involvement of p38 MAPK pathway in the phosphorylation of endothelial LSP1. Our data provide mechanistic evidence that the ICAM-1-mediated adhesion mechanism during leukocyte-endothelial cell interactions is essential for the phosphorylation of endothelial LSP1.

4.0 ENDOTHELIAL CELL-EXPRESSED LSP1 MODULATES ENDOTHELIAL CELL MIGRATION AND NEUTROPHIL RECRUITMENT BY REGULATING VASCULAR PECAM-1 EXPRESSION

A major part of the data presented in this chapter were used in a research paper entitled "Endothelial LSP1 modulates extravascular neutrophil chemotaxis by regulating non-hematopoietic vascular PECAM-1 expression" published by Mokarram Hossain, Syed M. Qadri, Najia Xu, Yang Su, Francisco S. Cayabyab, Bryan Heit and Lixin Liu in *The Journal of Immunology*, 2015; 195: 2408–2416.

4.0 ENDOTHELIAL CELL-EXPRESSED LSP1 MODULATES ENDOTHELIAL CELL MIGRATION AND NEUTROPHIL RECRUITMENT BY REGULATING VASCULAR PECAM-1 EXPRESSION

On account of its predominantly cytosolic presence and its association with the cytoskeleton, leukocyte-expressed LSP1 was reported to be crucial in transmission of signals that regulate leukocyte polarization and motility [140]. On the contrary, nonhematopoietic endothelial-expressed LSP1 is localized primarily in the nucleus [136]. In neutrophils, LSP1 is phosphorylated by soluble chemoattractants and then colocalizes with F-actin, thus contributing to the stability of cell polarization during chemotaxis [34, 208]. A similar mechanism was observed in migrating dendritic cells which showed enhanced phosphorylation of LSP1 concomitant with increased actin association in response to the HIV viral protein gp120 [35]. Surprisingly, these mechanisms are not operative in activation of endothelial cell-expressed LSP1 which becomes phosphorylated following ICAM-1-mediated adhesive engagement, but not by cytokine or chemokine stimulation alone (Figure 3.2 and Figure 3.5).

Despite the existence of mounting evidence regarding differences between leukocyte-expressed *versus* endothelial cell-expressed LSP1, there is no report on the role of endothelial cell-expressed LSP1 in endothelial functions apart from involvement in leukocyte recruitment and vascular leakage. There is a large body of evidence that overexpression of LSP1 in normally LSP1-null cells (sf9 and CV1-COS) induces the formation of filamentous projection in these cells [13]. Abundant expression of LSP1 affects motility of monocyte-differentiated phagocytes primarily by impairing their actin reorganizing capacity. However, constitutive low level of LSP1 expression is in fact, helpful for their motility [200]. In this study, we have explored whether and how LSP1 expression modulates endothelial cell motility which is important in many diseases such wound healing, angiogenesis, vascular inflammation and cancer.

4.1 Endothelial cell-expressed LSP1 is important for endothelial cell migration

Endothelial cell migration is usually measured as single cells along a chemotactic gradient in a Transwell membrane or migration as a whole monolayer which similar to the *in vivo* endothelium using a scratch wound healing assay. First, we compared the migration rate of EE2 cells before and after LSP1 silencing using a scratch wound healing assay.

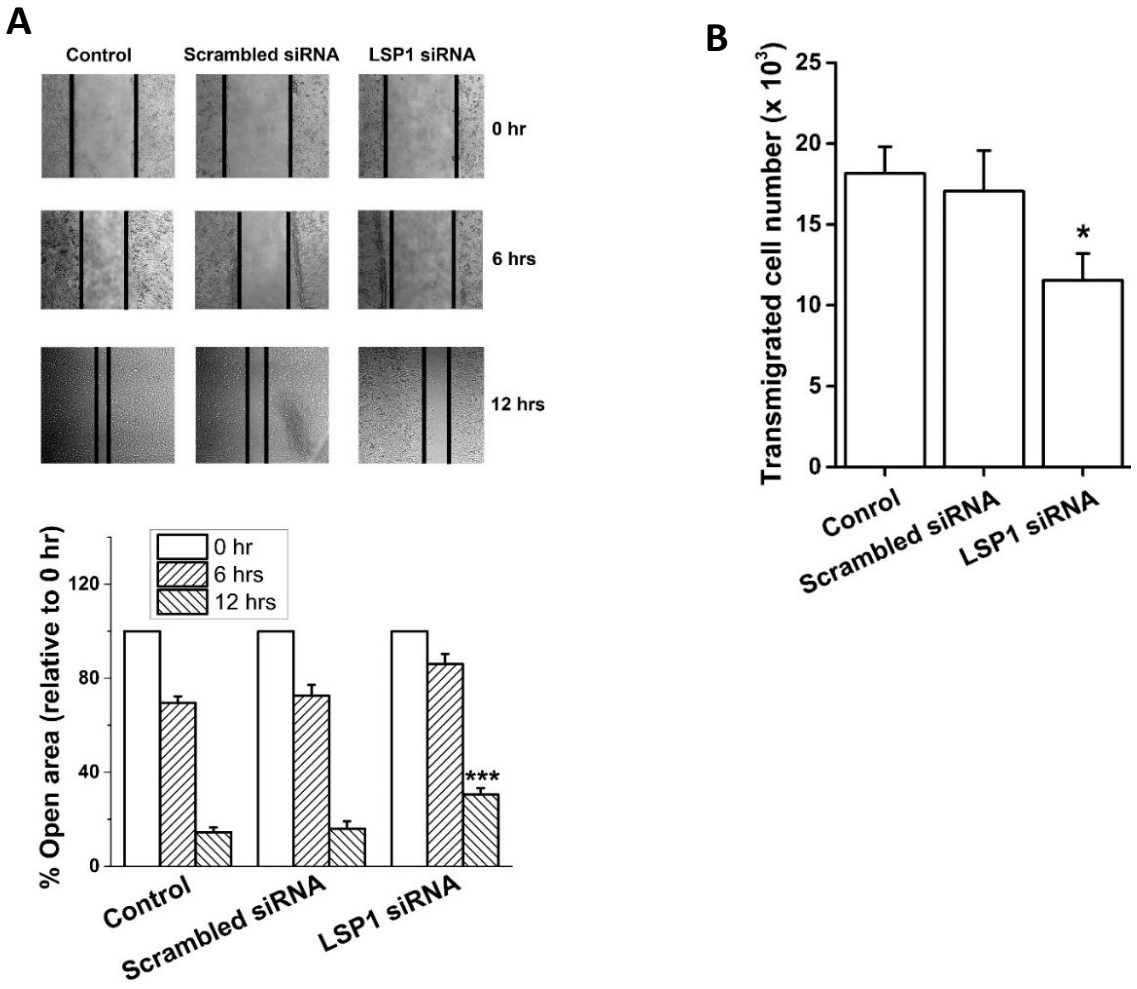

Figure 4.1: Role of LSP1 on endothelial cell migration.

(A) Confluent monolayers of EE2 cells before and after LSP1 silencing were wounded by making several straight scratches and wound closure was monitored for 24 hours. The percentage of open area at indicated time points was plotted. Beyond 12 hours, the wounds were completely healed, hence are not shown. Four independent wound healing migration assays were performed for each treatment groups. Data are means ± SD (n = 3). Significantly different from scrambled siRNA (***$P < 0.001$; ANOVA). **(B)** Number of migrated EE2 cells at the bottom of the polycarbonate membrane insert in response to serum gradient. Cells were counted in a hemocytometer after detaching them from the membrane by incubating the inserts in trypsin solution. LSP1 was silenced 24 hours prior to the migration assay. Data presented as means ± SD (n = 4). Significantly different from scrambled siRNA (*$P < 0.05$; ANOVA).

Closure of wounds took significantly longer time in LSP1-silenced cells compared to non-targeting siRNA-treated EE2 cells (Figure 4.1A). Closure of wounds requires cell migration as well as cell proliferation. In an attempt to distinguish whether LSP1-deficiency affects endothelial

cell motility, proliferation or both, we next measured the rate of migration of individual EE2 cells along serum gradient in Transwell systems. We noticed that EE2 cells treated with non-targeting siRNA transmigrated across the Transwell insert membrane of 3 μm pore size more efficiently than the LSP1-silenced EE2 cells (Figure 4.1B). Taken together, these results suggest that endothelial cell-expressed LSP1 promotes endothelial cell migration.

4.2 Deficiency of endothelial cell-expressed LSP1 plays a subtle role in the regulation of endothelial cell proliferation and apoptosis

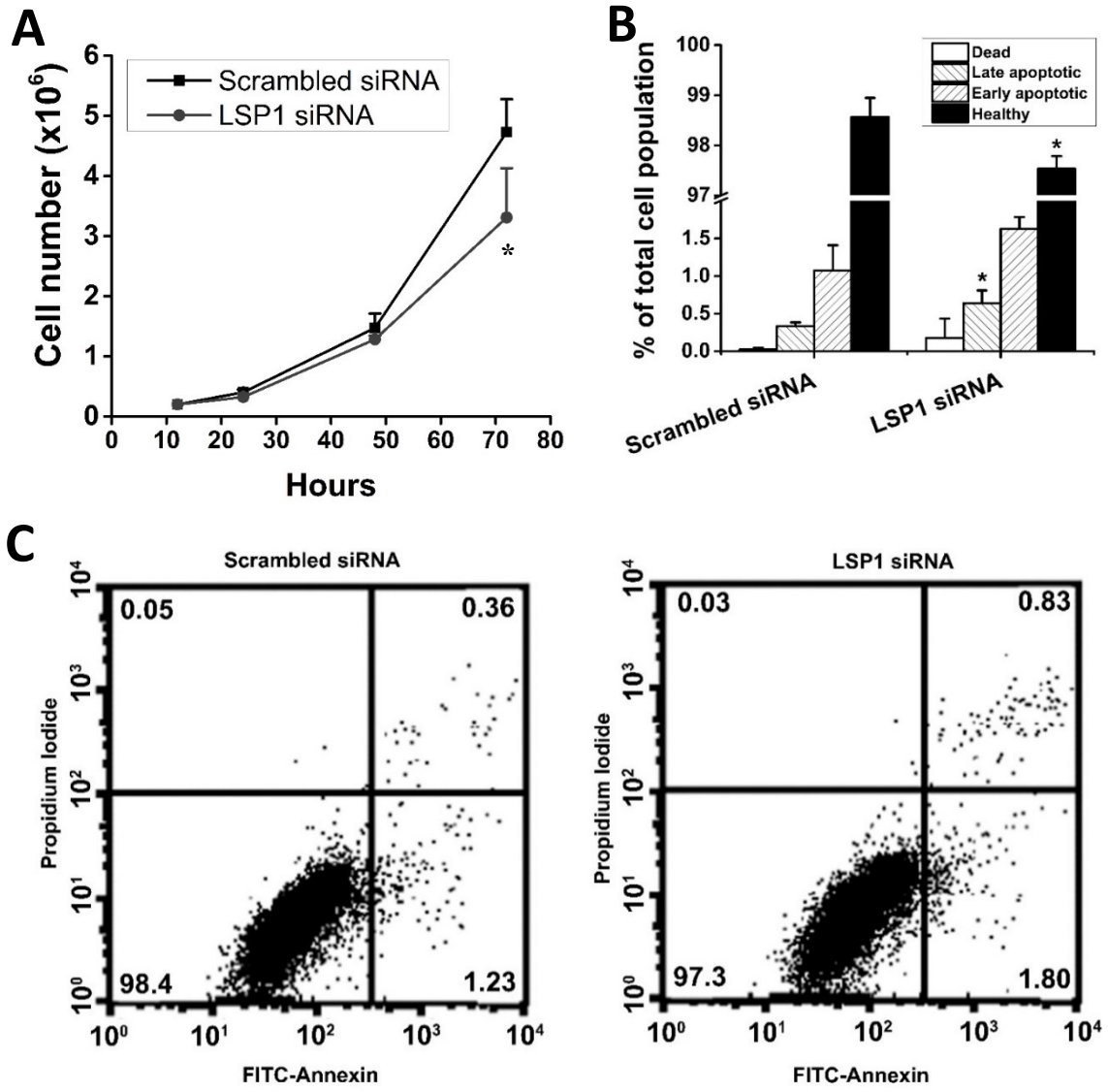

Figure 4.2: Role of LSP1 on endothelial cell proliferation. **(A)** EE2 cell monolayers were transfected with LSP1 or non-targeting silencing RNA at 60-80% confluency. After 12 hours, equal number (200,000) of these cells were subcultured in 100 mm cell culture dishes and the total cell number was counted using a hemocytometer at 24, 48 and 72 hours post-transfection. Three

independent cell proliferation assays were performed for each treatment groups. Data are means ± SD (n = 4). **(B-C)** EE2 cell monolayers were transfected with LSP1 or non-targeting silencing RNA at 60-80% confluency and grown for 48 hours. Spontaneous apoptosis of these cells was examined by flow cytometry using a FITC-Annexin and propidium iodide apoptosis detection kit. **(B)** Total count (% of total) of healthy, apoptotic and dead populations of EE2 cells presented as means ± SD (n = 3). Significantly different from scrambled siRNA (*$P < 0.05$; ANOVA). **(C)** Representative dot plots of FITC-Annexin vs propidium iodide-stained endothelial cells in non-targeting and LSP1 siRNA transfected EE2 cells. Healthy, apoptotic and dead cells counts (% of total) are indicated in the respective quadrants.

Regulators of endothelial cell migration often also modulate endothelial proliferation and apoptosis [212-214]. Furthermore, in B lymphocytes, LSP1 was shown to be a negative regulator of apoptosis ensuring better survival rate of WT B cells [215]. We have grown EE2 cells before and after LSP1 silencing for 72 hours to determine the role of LSP1 on endothelial cell proliferation. We found a consistent, subtle but significant reduction in the proliferation rate of LSP1-silenced EE2 cells compared to the non-targeting siRNA transfected EE2 cells (Figure 4.2A). To examine whether endothelial cell-expressed LSP1 provides similar protection to endothelial cells against apoptosis, we tested the rate of spontaneous apoptosis in EE2 cells before and after LSP1 silencing.

We found a subtle but a significantly increased tendency of endothelial cell apoptosis in LSP1-silenced EE2 cells (Figure 4.2B). Population of healthy cells was slightly smaller in LSP1-silenced endothelial cells (Figure 4.2B).

4.3 Endothelial cell-expressed LSP1 in neutrophil adhesion and transmigration

Negative regulatory role of neutrophil-expressed LSP1 on neutrophil adhesion, emigration, polarization and chemotaxis has been reported [147, 148]. On the contrary, the positive regulatory role of neutrophil-expressed LSP1 on neutrophil motility, chemotaxis, F-actin polarization and cytoskeletal rearrangement was observed by other groups [146, 208]. In another study, using knockout and chimeric mice, it was reported that LSP1 deficiency generally tends to decrease neutrophil adhesion [136, 216]. Because of these discrepancies, we investigated whether endothelial cell-expressed LSP1 participates in neutrophil adhesion. Using an *in vitro* assay, we measured the adhesion of WT neutrophils to WT and KO endothelial cells. As depicted in Figure 4.3A, incubation of chemokine-stimulated (KC or MIP-2, 100 nM) neutrophils with endothelial cells for 30 min remarkably enhanced the number of adherent neutrophils on WT and KO endothelial cells compared with neutrophil adhesion in the absence of chemokine stimulation.

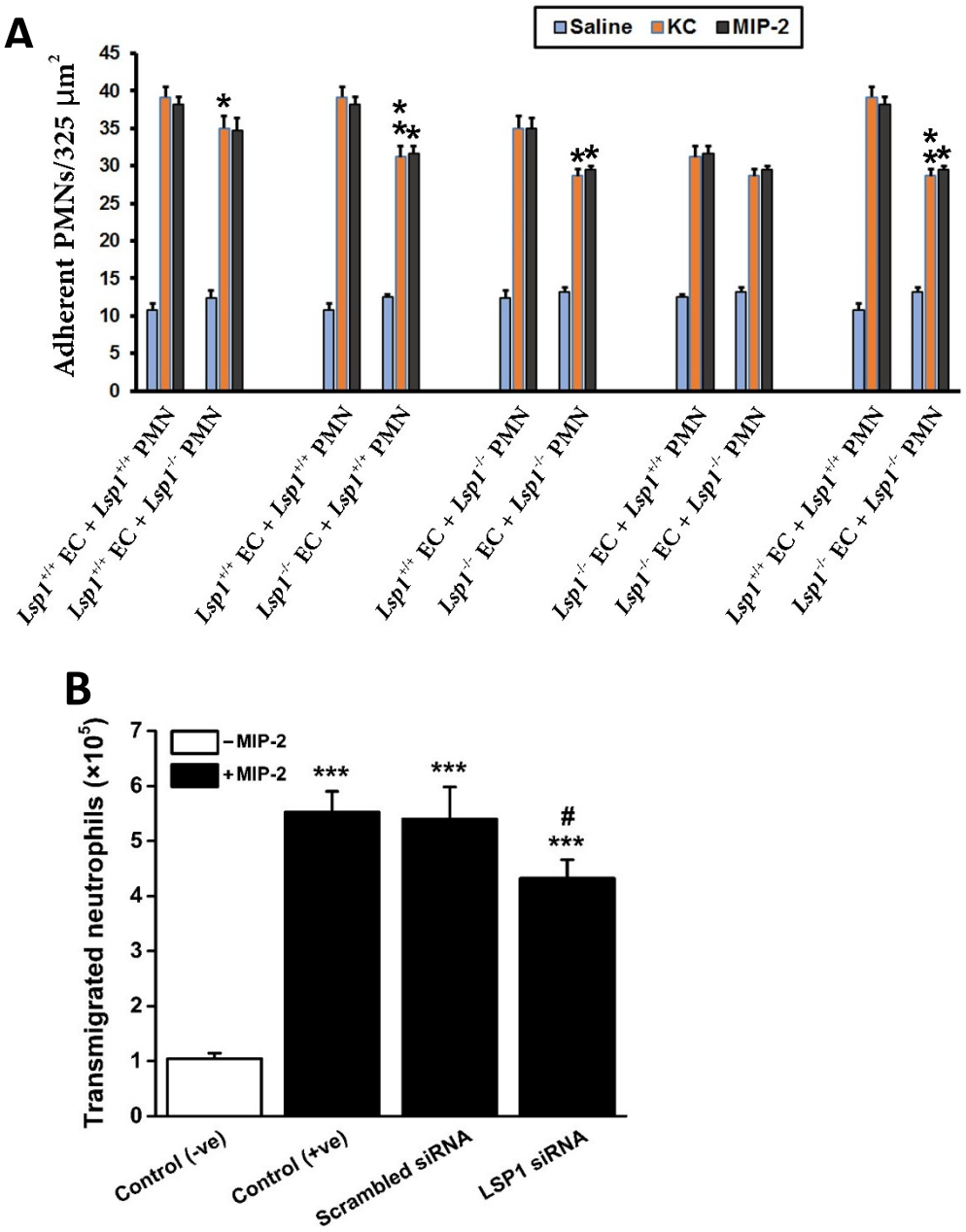

Figure 4.3: Role of endothelial cell-expressed leukocyte-specific protein 1 (LSP1) on neutrophil adhesion and transendothelial migration in vitro. **(A)** Number of adherent neutrophils (WT or KO; counted in a 325-µm^2 area) in the absence (control) and presence of stimulation with 100 nM KC or 100 nM macrophage inflammatory protein-2 (MIP-2) after 30 min of incubation on primary endothelial cells (EC) from WT and *KO* mice. Values are means ± SD (n = 3). Significant difference (*P < 0.05; **P < 0.01) due to LSP1 deficiency in neutrophil or endothelial cells (by ANOVA). **(B)** Number of migrated neutrophils across EE2 cell monolayers cultured on polycarbonate membrane inserts of a Transwell system. EE2 cells were treated with or without LSP1 siRNA or non-targeting siRNA for at least 24 hours before the transmigration assay. Transmigrated neutrophils were counted in a hemocytometer. Data presented as means ± SD (n = 4). Significantly different from negative control (***P < 0.001) or scrambled siRNA (#P < 0.05) (ANOVA).

The number of adherent neutrophils, however, was slightly, but significantly, lower on KO endothelial cells than on WT endothelial cells, suggesting that endothelial cell-expressed LSP1 plays a subtle but significant role in neutrophil adhesion (Figure 4.3A). Deficiency of neutrophil-expressed LSP1 also resulted in a subtle reduction in neutrophil adhesion which reached statistical significance only in KC treated neutrophils and only when these neutrophils were allowed to adhere onto WT endothelial cells (Figure 4.3A). We have also determined transmigration efficiency of neutrophils across EE2 cell monolayers cultured on Transwell inserts using MIP-2 as a chemotactic factor in a Transwell system. Transmigration assay was performed before or after LSP1 silencing to determine the role of LSP1 on neutrophil transendothelial migration. Results of the transmigration assays (Figure 4.3B) is consistent with previously reported *in vivo* results [136].

4.4 LSP1 deficiency affects PECAM-1 expression in the endothelium

Endothelial cell migration is crucial for angiogenesis in many health and pathologic conditions [8]. In angiogenesis, endothelial cells break up intercellular contacts, proliferate and migrate across the peri-endothelial barrier usually in response to many angiogenic factors [8, 55]. Numerous reports, albeit contradictory, are available highlighting the role of PECAM-1 on endothelial cell migration, proliferation and angiogenesis [213, 217-220]. Like LSP1 (Figure 4.5), PECAM-1-deficiency was also reported to cause dysregulation of endothelial permeability [221]. Using bone marrow transplanted chimeric mice, we observed that the expression of α_6 and β_1 integrins on the surface of neutrophils transmigrated across KO endothelium was dramatically reduced compared to neutrophils transmigrated across WT endothelium (Figure 4.4). Expression of $\alpha_6\beta_1$ integrins on the surface of transmigrated neutrophils is known to be modulated by homophilic PECAM-1 interactions during neutrophil-endothelial cell interactions [54]. All of these results indicate that LSP1 and PECAM-1 are functionally connected in endothelial cells. Hence, we hypothesized that the deficiency of endothelial cell-expressed LSP1 may lead to deranged PECAM-1 expression in endothelial cells. Accordingly, we designed *in vivo* and *in vitro* experiments to explore whether endothelial cell-expressed or neutrophil-expressed LSP1 modulates vascular PECAM-1 expression. Using fluorescence confocal imaging, we visualized the cremasteric microvasculature to examine vascular and neutrophil PECAM-1 expression in WT and KO mice. As depicted in Figure 4.5A, KO mice had a remarkably decreased level of microvascular PECAM-1 expression as compared to WT mice. Leukocyte PECAM-1-dependent fluorescence in cremasteric venule was, however, similar in both WT and KO mice. These results

indicate that endothelial cell-expressed LSP1 regulates endothelial PECAM-1 expression whereas neutrophil-expressed LSP1 does not regulate PECAM-1 expression in neutrophils.

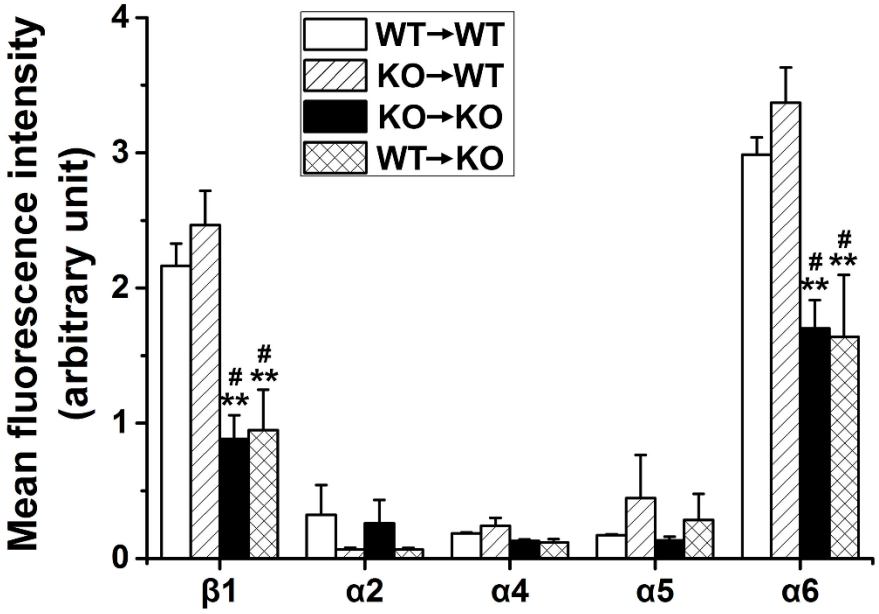

Figure 4.4: Expression of α6β1 integrin on the surface of transmigrated neutrophils is sensitive to endothelial cell-expressed LSP1. Means ± SEM (n = 3) of integrin-dependent fluorescence intensity quantified in transmigrated neutrophils obtained from peritoneal cavities of WT→WT, KO→WT, WT→KO, and KO→KO chimeric mice by peritoneal lavage 3 h after an i.p. injection of MIP-2. Significantly different from WT→WT (**P < 0.01) or from KO→WT (#P < 0.05) (ANOVA).

To corroborate these findings, we first detected PECAM-1 expression in mouse bone marrow neutrophils. As shown in Figure 4.5B, neutrophil PECAM-1 expression was not significantly different in either genotype (KO or WT mice). Using flow cytometry, we have also examined the PECAM-1 expression level in peripheral blood neutrophils and found that LSP1 deficiency did not affect PECAM-1 protein expression in circulating neutrophils (Figure 4.5C). These results confirms that leukocyte-expressed LSP1 does not regulate PECAM-1 expression. To further evaluate the cell-specific regulation of PECAM-1 expression by LSP1 and to determine whether LSP1 regulates other adhesion molecules in endothelial cells, we analyzed PECAM-1 and ICAM-1 protein expression in primary endothelial cells from WT and KO mice. As shown in Figure 4.5D, PECAM-1 but not ICAM-1 protein expression was significantly reduced in endothelial cells of LSP1 KO mice as compared to WT mice.

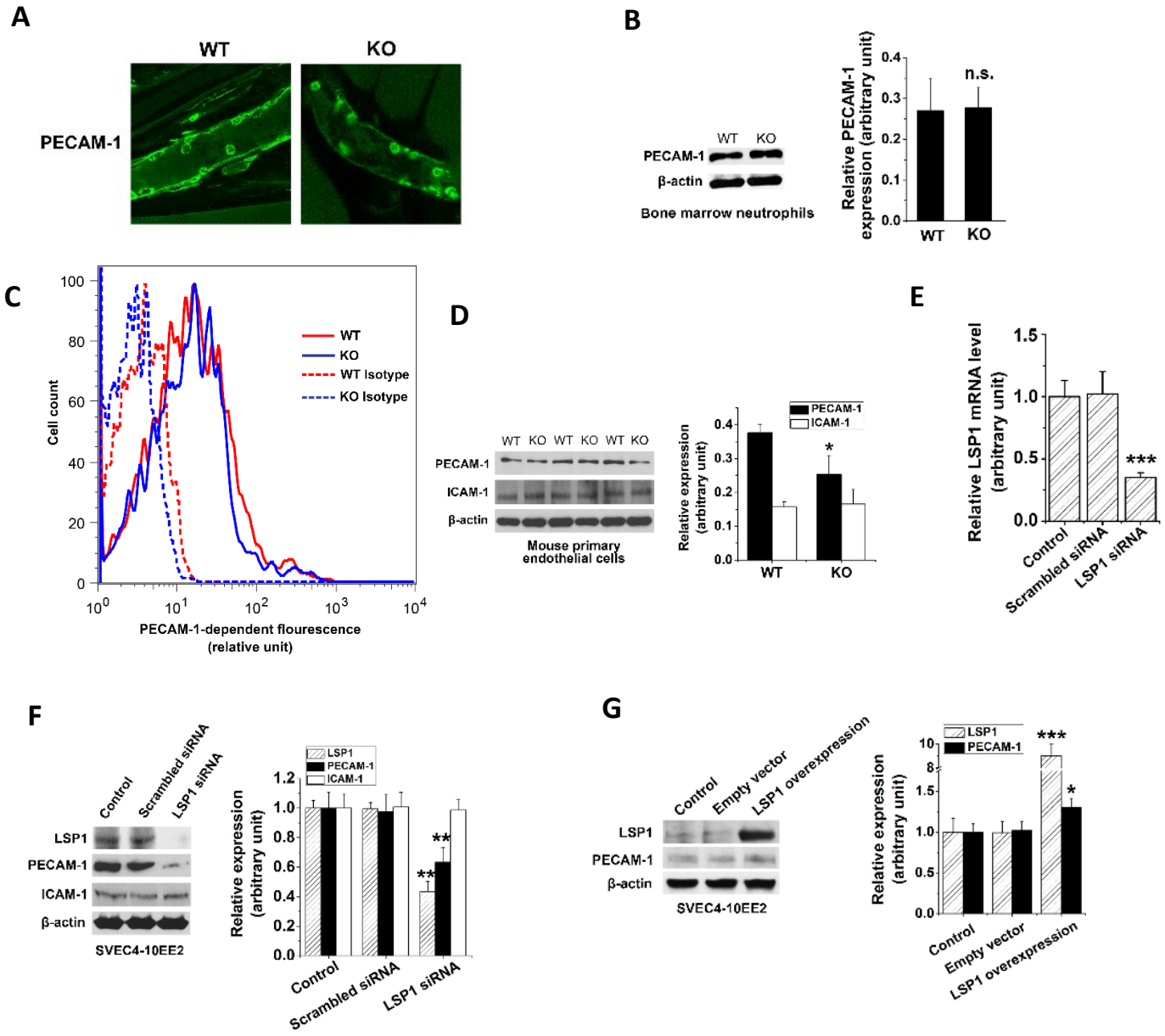

Figure 4.5: Endothelial cell-expressed LSP1-sensitive vascular PECAM-1 expression. **(A)** Confocal micrographs (n = 5) of PECAM-1-dependent fluorescence in cremasteric postcapillary venules of WT (left panel) and LSP1-deficient (KO, right panel) mice. **(B)** Representative original Western blot and means ± SD (n = 4) of PECAM-1 protein expression (relative to β-actin) determined in neutrophils isolated from bone marrows of WT and KO mice. n.s., not significant. **(C)** Original histograms of PECAM-1-dependent fluorescence in peripheral blood neutrophils from WT (red lines) or KO (blue lines) mice. Neutrophils were stained with FITC-conjugated PECAM-1 (solid lines) or isotype control (dashed lines) antibodies. **(D)** Original Western blot and means ± SD (n = 3) of PECAM-1 and ICAM-1 protein expression (relative to β-actin) determined in murine primary endothelial cells isolated from WT and KO mice. * ($P < 0.05$) from WT. **(E)** Relative LSP1 mRNA level (relative to β-actin) in EE2 endothelial cells in the absence (Control) or in the presence of treatment with negative control scrambled siRNA or LSP1-targeted siRNA. *** ($P < 0.001$) from scrambled siRNA treatment. **(F)** Representative original Western blot and

means ± SD (n = 4) of LSP1, PECAM-1 and ICAM-1 protein expression (relative to β-actin) determined in EE2 endothelial cells in the absence (Control) or in the presence of treatment with negative control scrambled siRNA or LSP1-targeted siRNA. ** ($P < 0.01$) from scrambled siRNA treatment. **(G)** Representative original Western blot and means ± SD (n = 4) of LSP1 and PECAM-1 protein expression (relative to β-actin) determined in EE2 endothelial cells in the absence (Control) or in the presence of transfection with murine pCMV-SPORT6-LSP1 plasmids or empty vector as negative control. * ($P < 0.05$) and *** ($P < 0.001$) from the control empty vector.

To support these data, we determined PECAM-1 protein expression after siRNA-targeted silencing of LSP1 in murine microvascular EE2 endothelial cells. As a result, siRNA-targeted silencing of endothelial cell-expressed LSP1 significantly reduced PECAM-1, without any effect on ICAM-1, expression in these murine endothelial cells (Figure 4.5F). Silencing LSP1 using gene targeted-siRNA significantly suppressed LSP1 protein expression indicating the experimental efficiency of LSP1 silencing in these endothelial cells (Figure 4.5E). Additional experiments explored whether overexpression of endothelial cell-expressed LSP1 modulates PECAM-1 expression. Figure 4.5G shows that overexpression of LSP1 in EE2 endothelial cells resulted in increased PECAM-1. Taken together, these data confirms that endothelial cell-expressed LSP1 selectively regulates PECAM-1 expression in endothelial cells whereas leukocyte-expressed LSP1 does not regulate PECAM-1 expression in leukocytes.

4.5 Endothelial cell-expressed LSP1 regulates vascular PECAM-1 expression via modulating GATA-2

We performed an additional series of experiments to disclose the underlying mechanisms of endothelial cell-expressed LSP1-sensitive PECAM-1 expression. As shown in Figure 4.6A, targeted siRNA silencing of LSP1 in murine EE2 endothelial cells significantly blunted PECAM-1, but not ICAM-1, mRNA levels suggesting that LSP1 transcriptionally regulates PECAM-1 expression in endothelial cells. To elucidate the transcriptional regulation of PECAM-1 by LSP1, we explored the participation of the transcription factor GATA-2 that is expressed in microvascular endothelial cells [222]. As shown in Figure 4.6B, GATA-2 expression in LSP1-deficient murine primary endothelial cells was significantly lower as compared to that in WT endothelial cells.

Furthermore, silencing LSP1 in EE2 endothelial cells significantly mitigated GATA-2 expression alluding to LSP1 sensitivity of endothelial GATA-2 expression (Figure 4.6C). Overexpression of LSP1 in these endothelial cells significantly increased the expression of GATA-2 (Figure 4.6D). To validate the role of LSP1-sensitive GATA-2 expression in selectively

regulating endothelial PECAM-1 expression, we silenced GATA-2 and analyzed adhesion molecule expression. Silencing endothelial GATA-2 significantly attenuated GATA-2 and PECAM-1, but not ICAM-1, protein expression in endothelial cells confirming the endothelial cell-specific regulation of PECAM-1 expression by GATA-2 (Figure 4.6E).

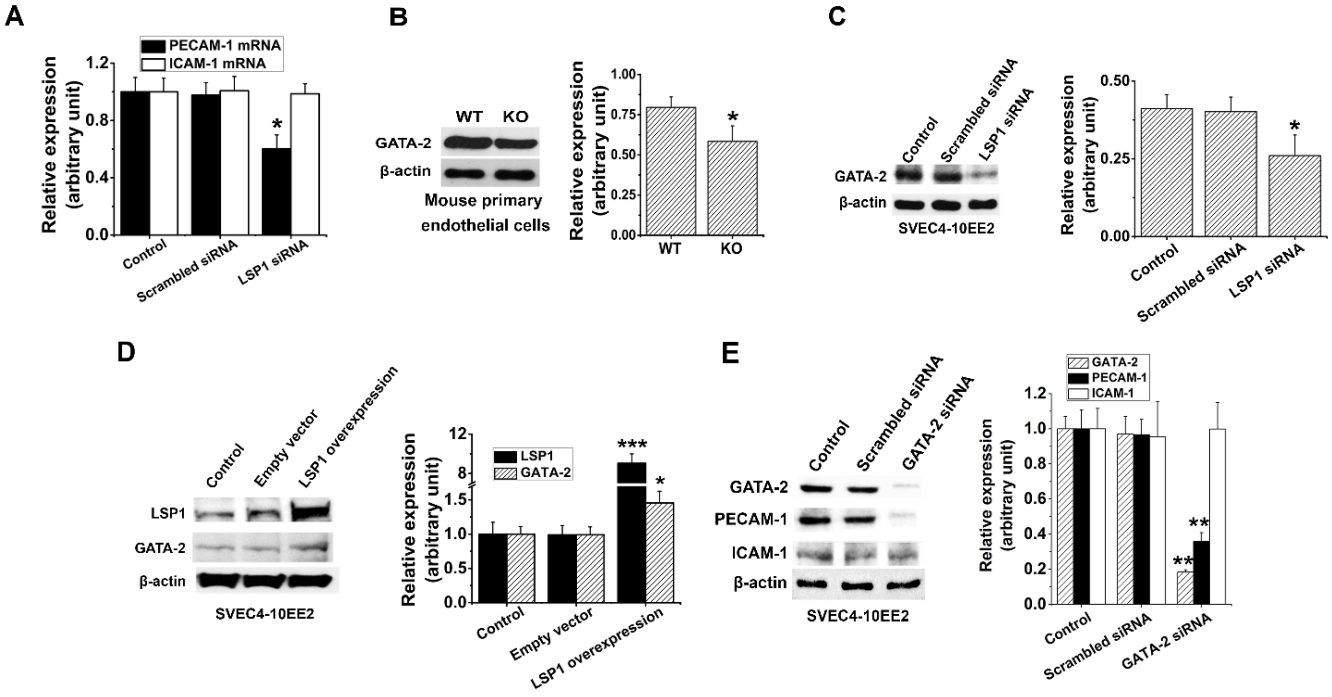

Figure 4.6: Endothelial cell-expressed LSP1-sensitive GATA-2 regulates PECAM-1 transcription. (**A**) Means ± SD of mRNA levels (n = 6) encoding PECAM-1 and ICAM-1 determined in EE2 endothelial cells in the absence (Control) or in the presence of treatment with negative control scrambled siRNA or LSP1-targeted siRNA. * ($P < 0.05$) from scrambled siRNA treatment. (**B**) Representative original Western blot and means ± SD (n = 4) of GATA-2 protein expression (relative to β-actin) determined in murine primary endothelial cells isolated from WT and KO mice. * ($P < 0.05$) from WT. (**C**) Representative original Western blot and means ± SD (n = 4) of GATA-2 protein expression (relative to β-actin) determined in EE2 endothelial cells in the absence (Control) or in the presence of treatment with negative control scrambled siRNA or LSP1-targeted siRNA. * ($P < 0.05$) from scrambled siRNA treatment. (**D**) Representative original Western blot and means ± SD (n = 4) of LSP1 and GATA-2 protein expression (relative to β-actin) determined in EE2 endothelial cells in the absence (Control) or in the presence of transfection with murine pCMV-SPORT6-LSP1 plasmids or empty vector as negative control. * ($P < 0.05$) and *** ($P < 0.001$) from the control empty vector. (**E**) Representative original Western blot and means ± SD (n = 4) showing GATA-2, PECAM-1 and ICAM-1 protein expression (relative to β-actin) determined in EE2 endothelial cells in the absence (Control) or in the presence of treatment with negative control scrambled siRNA or GATA-2-targeted siRNA. ** ($P < 0.01$) from scrambled siRNA treatment.

4.6 GATA-2 silencing mimics the effect of LSP1 silencing on endothelial cells functions

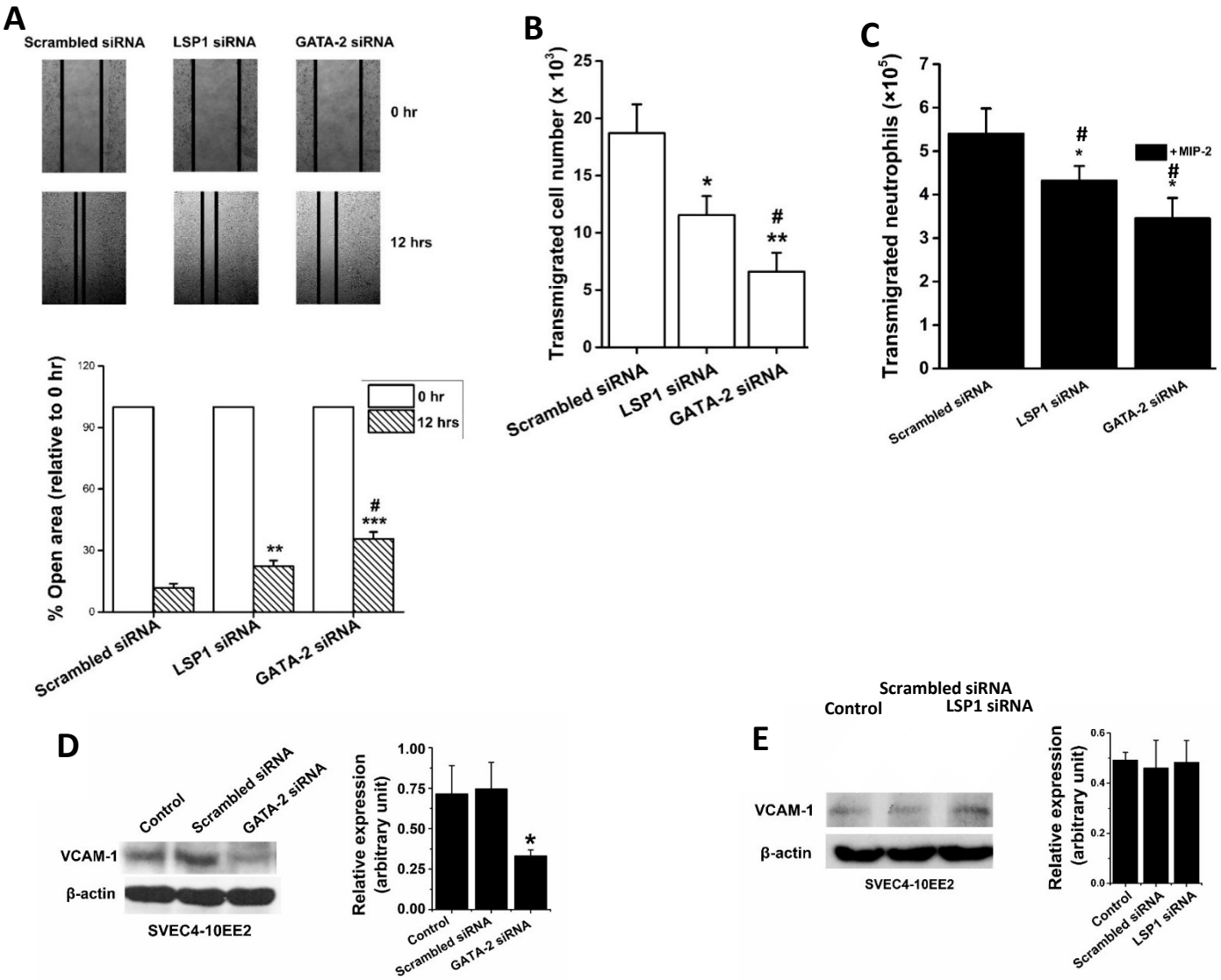

Figure 4.7: GATA-2 silencing mimics the effect of LSP1 silencing on endothelial cells functions. **(A)** Confluent monolayers of EE2 cells before and after LSP1 or GATA-2 silencing were wounded and wound closure was monitored for 24 hours. The percentage of open area at indicated time points was plotted. Beyond 12 hours, the wounds were completely healed, hence are not shown. Three independent wound healing migration assays were performed for each treatment groups. Data are means ± SD (n = 3). Significantly different from scrambled siRNA (**$P < 0.01$ and ***$P < 0.001$) or LSP1 siRNA (#P < 0.05) (ANOVA). **(B)** Number of migrated EE2 cells at the bottom of the polycarbonate membrane insert in response to serum gradient. Cells were counted in a hemocytometer after detaching them from the membrane by incubating the inserts in trypsin solution. LSP1 or GATA-2 was silenced 24 hours prior to the migration assay. Data presented as means ± SD (n = 3). Significantly different from scrambled siRNA (*$P < 0.05$

and **$P < 0.01$) or LSP1 siRNA (#P < 0.05) (by ANOVA). **(C)** Number of migrated neutrophils across EE2 cell monolayers cultured on polycarbonate membrane inserts of a Transwell system. EE2 cells were treated with or without LSP1, GATA-2 or non-targeting siRNA for at least 24 hours before the transmigration assay. Transmigrated neutrophils were counted in a hemocytometer. Data presented as means ± SD (n = 3). Significantly different from negative control (***$P < 0.001$) or scrambled siRNA (#P < 0.05) (by ANOVA). **(D)** Representative original Western blot and means ± SD (n = 5) showing VCAM-1 protein expression (relative to β-actin) in TNF-α-treated (4 h) EE2 endothelial cells in the absence (Control) or in the presence of treatment with negative control scrambled siRNA or GATA-2-targeted siRNA. * (P < 0.05) from scrambled siRNA treatment. **(E)** Representative original Western blot and means ± SD (n = 3) showing VCAM-1 protein expression (relative to β-actin) in TNF-α-treated (4h) EE2 endothelial cells in the absence (Control) or in the presence of treatment with negative control scrambled siRNA or LSP1-targeted siRNA.

Finally, to verify whether LSP1-deficiency-mediated impaired endothelial cell functions studied here such as endothelial cell migration and neutrophil transendothelial migration were due to reduced PECAM-1 expression, respective assays were repeated upon GATA-2 silencing in EE2 cells and the effect of LSP1 and GATA-2 deficiency was compared (Figure 4.7A-C). Both LSP1 and GATA-2 silencing had similar effect on the above-mentioned endothelial functions, however, the effect of GATA-2 silencing was more profound (Figure 4.7A-C). This difference may be attributed to the fact that in addition to regulating PECAM-1 expression, GATA-2 is decisive in endothelial-selective gene expression of endothelial nitric oxide synthase, endomucin and VCAM-1 [76-78, 216].

Recently, endothelial GATA-2 was shown to participate in regulating angiogenesis [79] and maintenance of vascular integrity [80]. Although GATA-2 regulated the transcription of both PECAM-1 and VCAM-1 in endothelial cells, we found that LSP1 selectively regulated GATA-2-mediated transcription of PECAM-1 but not ICAM-1 or VCAM-1 in these cells (Figure 4.5A, D, F-G; Figure 4.6 B-E &Figure 4.7 D-E).

4.7 Summary

In summary, these data provide a novel mechanistic insight into the cell-specific functions of endothelial cell-expressed LSP1 in regulating GATA-2-dependent vascular PECAM-1 expression. These altered PECAM-1 expression answers many questions, at least in part, regarding how LSP1 deficiency regulates endothelial functions such as maintenance of endothelial integrity, endothelial cell migration, neutrophil transendothelial migration and even expression of $\alpha_6\beta_1$ integrin on the surface of extravasated neutrophils.

5.0 PHOSPHORYLATED LEUKOCYTE-SPECIFIC PROTEIN 1 (LSP1) INTERACTS WITH MOESIN IN ENDOTHELIAL CELLS TO REGULATE VASCULAR PERMEABILITY

Data presented in this chapter were used in a manuscript entitled "ICAM-1 cross-linking-mediated LSP1-moesin interaction regulates endothelial permeability" to be submitted soon by Mokarram Hossain, Syed M. Qadri and Lixin Liu.

5.0 PHOSPHORYLATED LEUKOCYTE-SPECIFIC PROTEIN 1 (LSP1) INTERACTS WITH MOESIN IN ENDOTHELIAL CELLS TO REGULATE VASCULAR PERMEABILITY

Leukocyte-specific protein 1 (LSP1) expressed in both leukocytes and endothelial cells interacts with cytoskeletal proteins, regulates cytoskeletal rearrangement and is an important regulator of endothelial permeability [136, 139]. While endothelial LSP1 was shown to be important for histamine-induced endothelial hypermeability, it was also reported to prevent neutrophil transmigration-mediated vascular leakage [37, 136]. However, the mechanistic role of LSP1 in regulating endothelial permeability is yet to be elucidated. Leukocyte-endothelial cell interactions involve very complex interactions of many adhesion molecules and are capable of initiating a wide range of signaling cascades in endothelial cells. In this study, we explored the molecular mechanisms of how endothelial cell-expressed LSP1 modulates endothelial permeability in a stimulus-specific manner.

5.1 LSP1 interacts with moesin in HEK 293T and endothelial cells

It is well known that during neutrophil adhesion, ICAM-1 clustering controls the cytoskeletal structure of endothelial cells very precisely by interacting, activating and recruiting many proteins capable of reorganizing endothelial cytoskeleton such as ERM (ezrin/radixin/moesin) proteins, myosin II, β-actin and LSP1 ([44, 223]; Figure 3.5). All of these proteins are well known for regulating endothelial permeability usually by regulating contraction of endothelial cells. However, none of them can regulate cell shape independently; they either need to get activated by other signaling proteins or interact and collaborate with other proteins. ERMs are capable of remodeling the actin cytoskeleton, however, they lack F-actin cross-linking or branch-forming abilities [176]. Moreover, ERMs were reported to be abundantly present at both the uropod of T-lymphocytes and docking structures in endothelium [178, 179]. LSP1 was also reported to be necessary for endothelial dome formation during neutrophil transmigration [37].

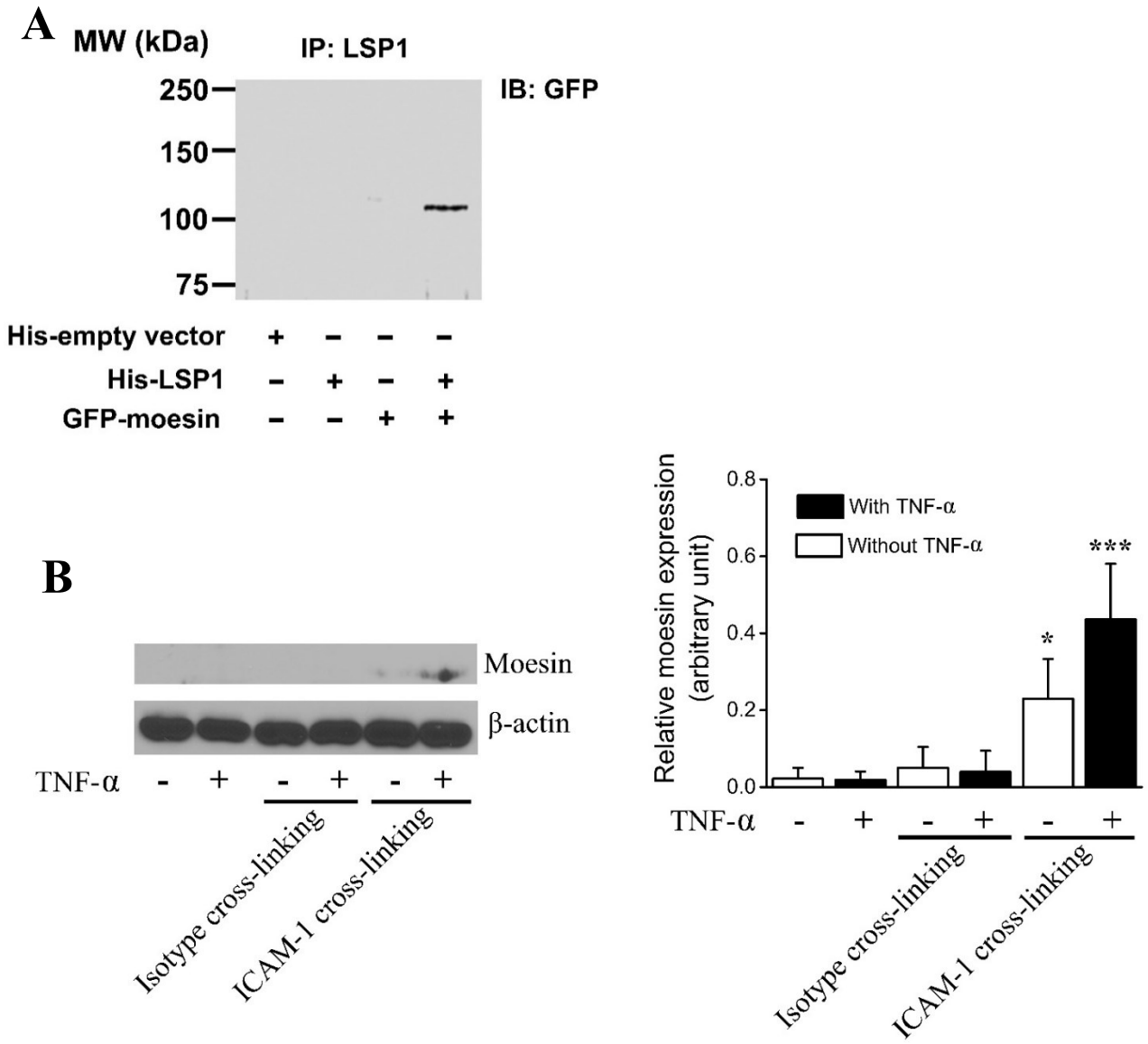

Figure 5.1: LSP1 interacts with moesin both in HEK 293T and endothelial cells. **(A)** HEK239T cells were transfected with His-LSP1, GFP-Moesin or co-transfected with His-LSP1 and GFP-Moesin. After 24 hours, His-LSP1 was immunoprecipitated with rabbit-α-LSP1 serum and immunodetected by an anti-GFP antibody. Representative blots from one of the three independent experiments are shown. **(B)** *Left panel*: EE2 cells were treated with or without TNF-α (4h) before cross-linking endothelial surface ICAM-1 with rat-α-ICAM-1 primary (30 min) and goat-α-rat secondary antibodies (30 min). After ICAM-1 cross-linking, cells were lysed, LSP1 was immunoprecipitated with rabbit-α-LSP1 serum and immunoblotted with anti-moesin antibody. Representative blots from one of the three independent experiments are shown. *Right panel*: densitometric analysis of the relative abundance of moesin over β-actin. Data are means ± SD (n = 3). * indicates significant difference ($P < 0.05$) in comparison to control (ANOVA).

Therefore, we explored whether LSP1 interacts with moesin, the major (> 90% or the total ERM proteins) endothelial ERM protein. We overexpressed both His-LSP1 and GFP-moesin in HEK 293T cells. Pull-down of His-tagged LSP1 protein following immunoblotting with anti-GFP antibody provided the indication of this novel interaction (Figure 5.1A). In endothelial cells, we first explored whether constitutively expressed LSP1 and moesin interact with each other. To this end, we treated endothelial cells with or without a wide range of stimuli which were previously reported to activate endothelial LSP1 or moesin to address whether the potential interaction is stimuli-specific or not. Our results in Figure 5.1B show that constitutively expressed LSP1 and moesin interact in endothelial cells. However, unlike LSP1- and moesin-overexpressing HEK 293T cells, this interaction takes place only upon ICAM-1 cross-linking (Figure 5.1B) which is largely regarded as equivalent to leukocyte adhesion onto endothelial cells [224]. These findings indicate that LSP1 interacts with moesin and LSP1 phosphorylation is probably required for this interaction because ICAM-1 cross-linking is necessary for LSP1 (Figure 3.5) but not for moeisn phosphorylation.

5.2 LSP1-moesin interaction enhances moesin but not LSP1 phosphorylation

Protein-protein interactions often regulate phosphorylation of the interacting protein(s) either by acting as a kinase or a scaffold that can bring a substrate to its kinase in close proximity [225, 226]. Therefore, we investigated whether this interaction affects phosphorylation level of LSP1, moesin or both of them. In a series of experiments, we cross-linked ICAM-1 in TNF-α-treated endothelial cells which were transfected with or without silencing RNA targeting LSP1 or moesin. Then, we compared the relative phosphorylation of LSP1 before and after moesin silencing and moesin phosphorylation before and after LSP1 silencing.

Figure 5.2A clearly shows that this interaction does not affect LSP1 phosphorylation at all indicating LSP1 phosphorylation to be either upstream or independent of this interaction. However, LSP1-moesin interaction is capable of increasing the level of moesin phosphorylation, even though, it is not required for moesin phosphorylation in endothelial cells (Figure 5.2B). Phosphorylation of endothelial LSP1 is quite unique and requires ICAM-1 clustering. Moesin phosphorylation, on the other hand, is easily achieved by treating the cells with TNF-α alone. In our experiment design, cells were treated with TNF-α before ICAM-1 was cross-linked. TNF-α pretreatment was sufficient to phosphorylate moesin (Figure 5.2B) whereas ICAM-1 cross-linking was necessary for LSP1 phosphorylation (Figure 3.5).

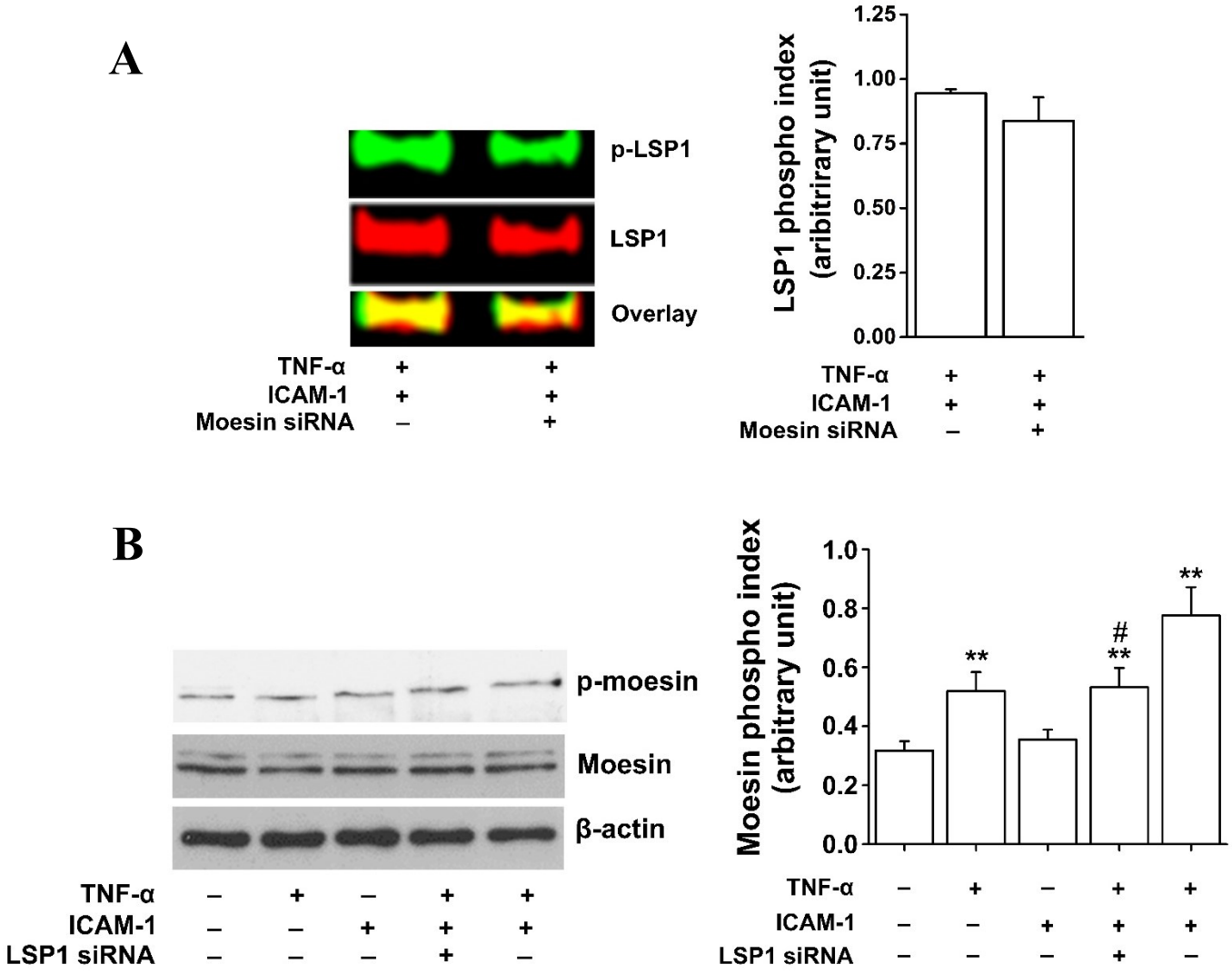

Figure 5.2: LSP1-moesin interaction enhances moesin but not LSP1 phosphorylation. **(A)** Left panel: original dual immunoblots (representative of 3 similar experiments) demonstrating expression of phosphorylated LSP1 (p-LSP1, Alexa Fluor 488, green), total LSP1 (LSP1, Alexa Fluor 647, red), and overlap of phosphorylated LSP1 and total LSP1 (p-LSP1 + LSP1, yellow) in moesin silenced or WT endothelial cells after ICAM-1 cross-linking. Right panel: densitometric analysis of the abundance of phosphorylated LSP1 relative to total LSP1 in moesin silenced or WT endothelial cells after ICAM-1 cross-linking. Values are means ± SD (n = 3). **(B)** Left panel: original immunoblots (representative of 3 similar experiments) demonstrating expression of phosphorylated moesin (p-moesin), total moesin (moesin) and β-actin in LSP1 silenced or WT endothelial cells before or after treatment with TNF-α, or ICAM-1 cross-linking before or after TNF-α treatment. Right panel: densitometric analysis of abundance of phosphorylated moesin and total moesin relative to β-actin in LSP1 silenced or WT endothelial cells before or after treatment with TNF-α, or ICAM-1 cross-linking before or after TNF-α treatment. Values are means ± SD (n = 3). Significantly different from untreated cells (**P < 0.01) or from TNF-α + ICAM-1 cross-linking before moesin silencing (#P < 0.05) (ANOVA).

LSP1 phosphorylation was unaffected by the presence or absence of its binding partner moesin (Figure 5.2A) suggesting LSP1 phosphorylation to be independent or upstream of this interaction. Absence of LSP1, however, significantly reduced moesin phosphorylation (Figure 5.2B) even though LSP1 is not a kinase itself. This observation indicates that LSP1 acts as a scaffold or at least a component of a scaffold that can recruit both moesin and the upstream effector kinase(s) necessary for moesin phosphorylation. In dendritic cells LSP1 was indeed shown to interact with scaffolds KSR1 and CNK in DC-SIGN signalosome where LSP1 is required for the recruitment of Raf-1 and its effector LARG and Rho A and subsequent Raf-1 phosphorylation upon mannose-expressing microbial challenge [165]. ERM proteins are well known substrates of Rho kinase, however, whether LSP1 aids moesin phosphorylation by bringing them together is yet to be explored.

5.3 Both p38 MAPK and Rho kinase pathways are involved in LSP1-moesin interaction

Activation of endothelial LSP1 is mainly mediated by p38 MAPK whereas moesin can be activated by p38 MAPK and Rho kinases. Thus, we investigated whether either of these two pathways is involved in the regulation of LSP1-moesin interaction and if this interaction is upstream or downstream of these kinases in endothelial cells. In this regard, we treated the cells with pharmacological inhibitors of p38 MAPK (SB 203580) and Rho kinase (Y-27632 and C3 transferase) before measuring this interaction.

From our results, it is evident that inhibition of either of these pathways blunts this interaction significantly (Figure 5.3). However, p38 MAPK pathway plays a more prominent role in this interaction (Figure 5.3). These results suggest that both p38 MAPK and Rho kinase pathways regulate this interaction and are upstream of this interaction indicating that LSP1 and moesin interact with each other upon activation. Phosphorylation may change the physico-chemical properties, stability, kinetics, and dynamics of a protein [227]. By activating or inhibiting protein functions, phosphorylation regulates many cellular processes. Recent studies involving phosphoproteomic analyses have revealed that the majority of mammalian proteins are modified by transient phosphorylation [226, 228, 229] which suggests that the regulatory property of protein phosphorylation to be very extensive. Phosphorylation is also known to regulate the nature and the strength of protein-protein interactions either directly when phosphorylation takes place at a site close to the binding site or via allosteric mechanism when phosphorylation occurs at site distant

from the binding site [226, 230, 231]. Phosphorylation of LSP1 (Figure 3.5) and LSP1-moesin interaction both require ICAM-1 cross-linking. Moreover, LSP1-moesin interaction enhances moesin phosphorylation without affecting LSP1 phosphorylation at all. This observation confirms that ICAM-1 cross-linking-mediated LSP1 phosphorylation is necessary for LSP1-moesin interaction in endothelial cells.

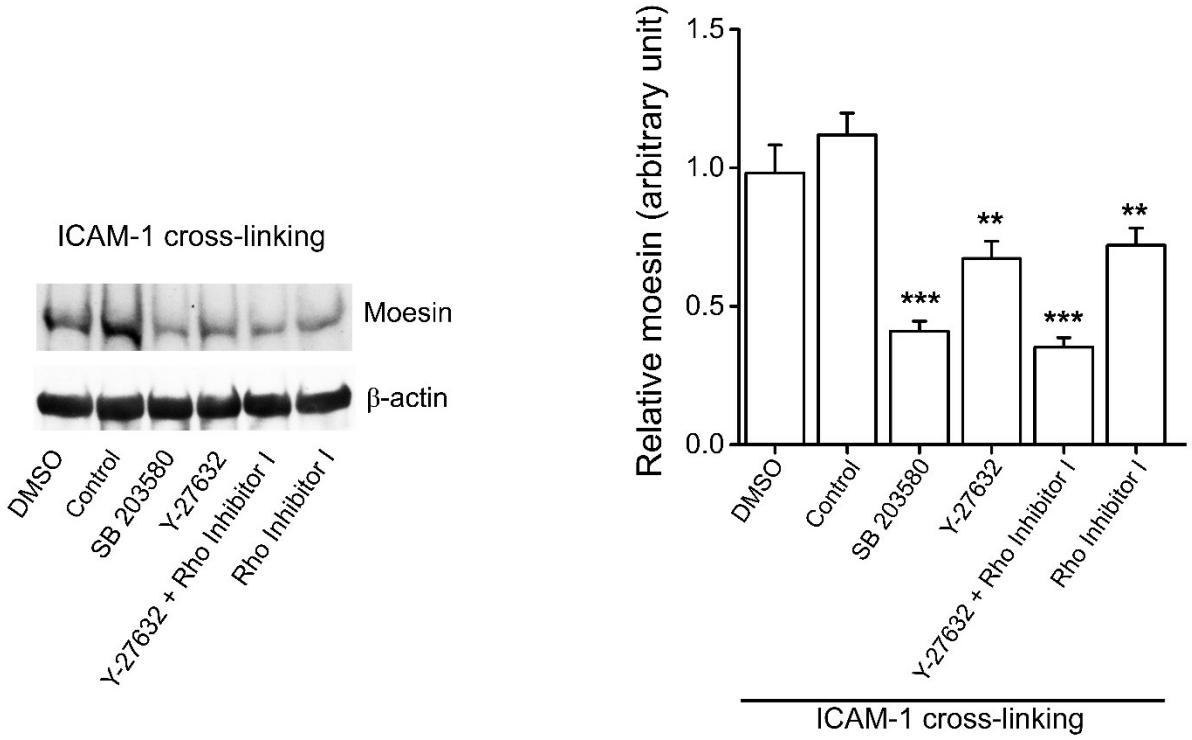

Figure 5.3: Both p38 MAPK and Rho kinase pathways regulate LSP1-moesin interaction. Left panel: endothelial cells were treated with TNF-α (4h) before cross-linking endothelial surface ICAM-1 with rat-α-ICAM-1 primary (30 min) and goat-α-rat secondary antibody (30 min). Cells were treated with SB 203850, Y-27632 and Rho inhibitor I (C3 transferease) right before ICAM-1 cross-linking. After ICAM-1 cross-linking cells were lysed, LSP1 was immunoprecipitated with rabbit-α-LSP1 serum and immunoblotted with anti-Moesin antibody. Right panel: densitometric analysis of the relative abundance of moesin over β-actin. Data are means ± SD (n = 3). Significant difference from control (**P < 0.01; ***P < 0.001) in comparison to control (ANOVA).

5.4 LSP1-moesin interaction helps regulate endothelial permeability

Endothelial LSP1-deficiency was reported to impair histamine-induced endothelial permeability *in vivo* which is mediated by endothelial cell contraction [136]. However, neutrophil migration-induced endothelial hypermeability usually mediated by endothelial injury was significantly higher in LSP1-deficient mice [37]. This clear contrast in the role of LSP1 in

stimulus-specific endothelial hypermeability and the well-known ability of both LSP1 and moesin to remodel cellular shape (cytoskeleton) prompted us to determine the effect of LSP1-moesin interaction on endothelial permeability. TNF-α treatment resulted in a steady increase in endothelial permeability of FITC-albumin in EE2 cells before LSP1 or moesin silencing (Figure 5.4A).

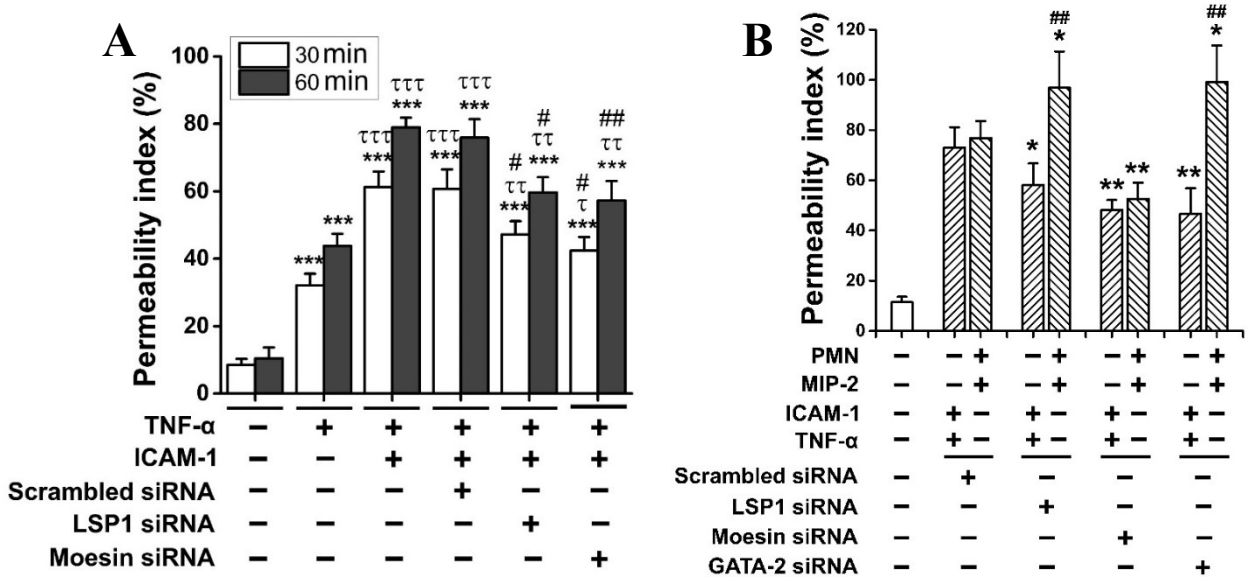

Figure 5.4: Lack of LSP1-moesin interaction impairs ICAM-1 cross-linking-mediated endothelial hepermeability. EE2 cells were grown to confluence on Transwell insert membranes before or after silencing the genes of interests. Cells were treated with or without TNF-α (20 ng/ml; 4 hours) before ICAM-1 cross-linking for 30-60 min **(A-B)** or neutrophils were allowed to transmigrate across the endothelial monolayer along a MIP-2 gradient for 60 min **(B)**. FITC-albumin (200 μg/ml) was placed in the upper chamber, and samples of medium from both the upper and lower chambers were then taken at indicated time points. FITC-albumin contents in the upper and lower chambers was measured in a plate reader in the beginning and end of each time points. Data are means ± SD (n = 3-4). **(A)** Significant difference from control (***P < 0.001), TNF-α control ($^{\tau}P < 0.05$; $^{\tau\tau}P < 0.01$; $^{\tau\tau\tau}P < 0.001$) or scrambled siRNA treatment ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$) (ANOVA). **(B)** Significant difference from scrambled siRNA treatment (*P < 0.05; **P < 0.01), ICAM-1 cross-linking ($^{\#\#}P < 0.01$) (ANOVA).

However, this TNF-α-mediated hypermeability was less prominent in LSP1- or moesin-silenced EE2 cells. ICAM-1 cross-linking following TNF-α-treatment significantly increased permeability of EE2 cells before LSP1- or moesin-silencing but not after silencing LSP1 or moesin (Figure 5.4A). Interestingly, when neutrophils were allowed to transmigrate across these EE2 cells in place of ICAM-1 cross-linking after TNF-α-treatment, permeability increase was significantly

higher in LSP1- and GATA-2- but not moesin-silenced cells compared to the non-targeting siRNA-treated cells (Figure 5.4B). It is known that vascular leakage induced by different type of stimuli is mediated by different molecular mechanisms. These complicated findings suggest that LSP1 plays a differential role likely via different pathways in regulating vascular leakage. Hypermeability inducing agents capable of modulating cell shape such as histamine and ICAM-1 clustering utilize LSP1-moesin interaction and related pathway. Taken together, these observations confirm that LSP1-moesin interaction plays a crucial role in LSP1-mediated regulation of endothelial permeability. On the other hand, neutrophil-mediated endothelial injury causes direct endothelial cell detachment by damaging the endothelial junctional proteins like VE-cadherin and PECAM-1 [6]. Because we know that LSP1-deficient endothelial cells express significantly lower level of PECAM-1 (Figure 4.5), PECAM-1-deficiency makes the LSP1-deficient cells more vulnerable to injury-mediated hypermeability.

5.5 Summary

Both LSP1 and moesin are capable of interacting with cellular actin cytoskeleton and remodel it. Here, we report for the first time that LSP1 and moesin interact with each other in endothelial cells. LSP1-moesin interaction is regulated by p38 MAPK and Rho kinase pathways and phosphorylation of LSP1 is necessary for this interaction. This interaction plays a major role in regulating endothelial permeability primarily by modulating endothelial cell contraction.

6.0 SUMOYLATION PROTECTS ENDOTHELIAL LSP1 FROM UBIQUITINATION-MEDIATED PROTEASOMAL DEGRADATION AND PROMOTES ITS NUCLEAR EXPORT

Data presented in this chapter were used in a manuscript entitled "SUMOylation protects endothelial LSP1 from ubiquitination-mediated proteasomal degradation and helps its nuclear export" (currently in revision in The Journal of Biological Chemistry) prepared by Mokarram Hossain, Jiannan Huang, Francisco S. Cayabyab and Lixin Liu.

6.0 SUMOYLATION PROTECTS ENDOTHELIAL LSP1 FROM UBIQUITINATION-MEDIATED PROTEASOMAL DEGRADATION AND PROMOTES ITS NUCLEAR EXPORT

The mouse LSP1, with 330 amino acids, is predicted to be 37 kDa in molecular weight [130]. However, in Western blotting, LSP1 has always been detected at a much higher molecular weight range (>50 kDa) both in leukocytes and endothelial cells [130, 141, 143, 182]. Posttranslational modifications often result in uplift of molecular weight of the modified proteins [81, 183] and are linked to compartmentalization and translocation of many proteins [88, 184, 185]. This discrepancy in LSP1 molecular weight (15 kDa) and its characteristic distribution pattern in endothelial cells and nucleus-to-extranuclear translocation upon endothelial stimulation with TNF-α [37] indicate 'SUMOylation' to be the most likely posttranslational modifications of LSP1. Scarcity of information regarding posttranslational modifications of LSP1, despite having multiple indications like the ones mentioned above, prompted us to explore the possibility of LSP1 SUMOylation in endothelial cells and determine whether SUMOylation-mediates changes in LSP1 function.

6.1 LSP1 is modified by SUMO1 in endothelial cells and HEK 293T cells

Even after having multiple indications (as mentioned above), nothing is known about posttranslational modifications of endothelial cell-expressed LSP1. Using anti-LSP1 rabbit sera, we detected three distinct bands at 37 kDa, 45 kDa and 52 kDa molecular weight range in mouse primary endothelial cells isolated from lungs (LVEC) or hearts (HVEC) and in untransfected as well as LSP1-overexpressed endothelial cell line (EE2) cells (Figure 6.1A). This indicates post-translational modifications of LSP1 or non-specific nature of the antibody. To address this issue, we excised the respective bands from Coomassie blue-stained gel and performed mass spectrometric analysis. Mass spectrometry confirmed the presence of LSP1 in 37 kDa band (Mowse/ions score: 89; queries matched: 2; sequence coverage: 7%; p*I*/mol wt: 4.7/36.7) and 52 kDa band ((Mowse/ions score: 98; queries matched: 3; sequence coverage: 11%; p*I*/mol wt: 4.7/36.7). Ion score is −10×log (*P*), where *P* is the probability that the match is a random event. Individual ion score >32 indicates identity or extensive homology ($P < 0.05$)). However, mass spectrometric analysis of the 45 kDa band was unsuccessful. Immunoblotting of pulled-down His-tagged proteins from His-LSP1- and SUMO1-overexpressed EE2 cells using monoclonal anti-

SUMO1 antibody revealed a single band at 52 kDa indicating this band to be SUMO1-LSP1 conjugate (Fig. 1A).

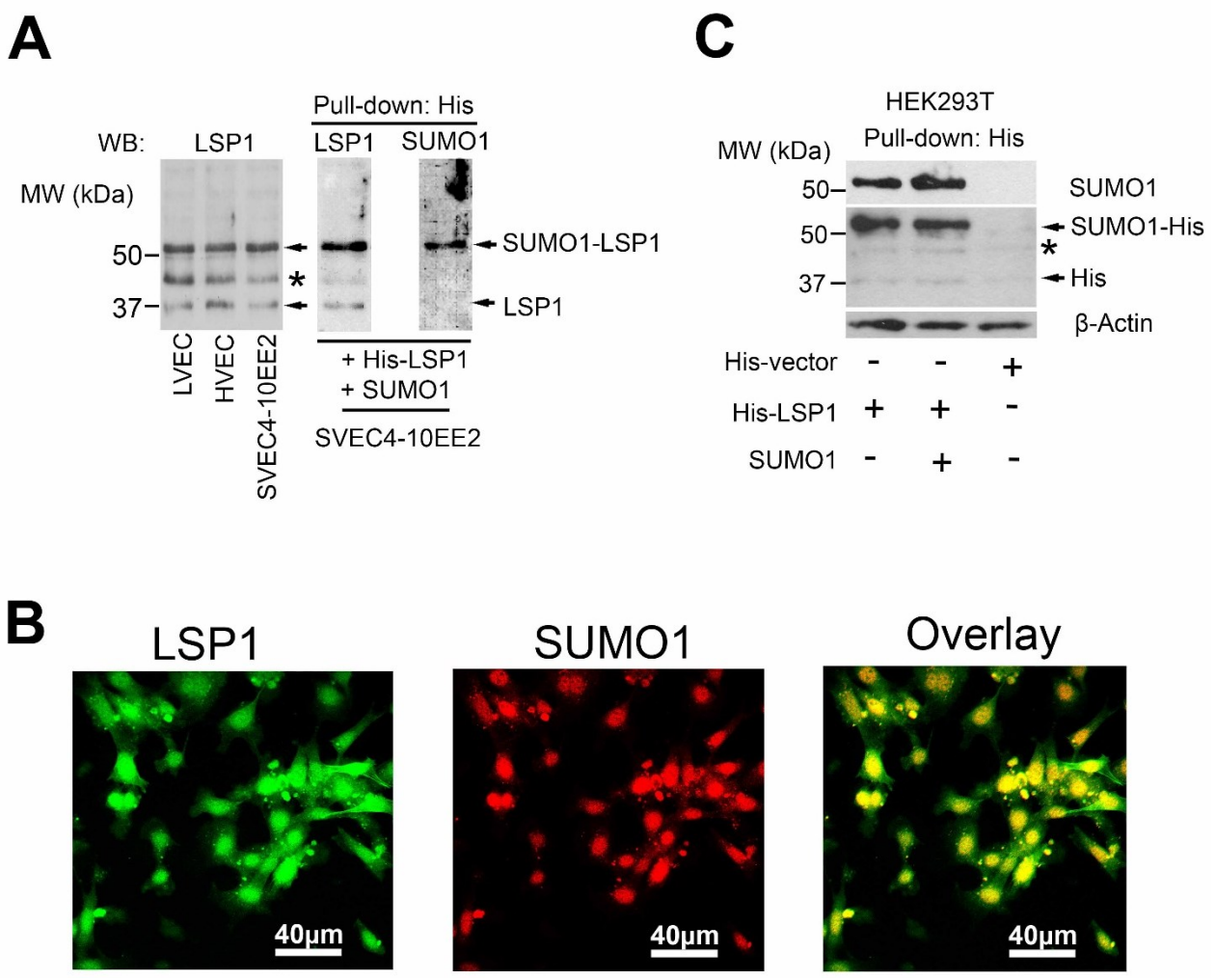

Figure 6.1: LSP1 is modified by SUMO1. **(A)** Representative (n = 3) original Western blots showing LSP1 protein expression in murine primary vascular endothelial cells isolated from heart (HVEC) and lungs (LVEC) of 129/SvJ mice and untransfected murine endothelial cell line (EE2) cells (left); LSP1 (middle) and SUMO1 (right) protein expression in pulled-down His-tagged proteins from His-LSP1- and SUMO1-overexpressed EE2 cells. Arrow indicates LSP1 or SUMO1-LSP1 conjugate and * indicates an unknown signal. **(B)** Representative (n = 3) confocal images of LSP1 (Green; left), SUMO1 (Red; middle) and overlay (right) in LSP1- and SUMO1-overexpressed EE2 cells. **(C)** Representative (n = 3) original Western blot showing SUMO1 (top) and His (middle) protein expression in pulled-down His-tagged proteins and β-actin (bottom) protein expression in the whole cell lysate from His-LSP1- or His-LSP1 and SUMO1-overexpressed HEK 293T cells. Arrow indicates His or SUMO1-His conjugate and * indicates an unknown signal.

For a SUMOylated protein, relative abundance of the SUMOylated fraction is usually quite low though the opposite report is also available [83]. This relative abundance may also vary depending on the cell lysis procedure and the type of lysis buffer being used [83]. GFP-LSP1 and SUMO1 co-localization was also confirmed by confocal microscopy of GFP-LSP1 and SUMO1 co-transfected EE2 cells (Figure 6.1B).

Moreover, we examined SUMOylation of LSP1 with SUMO1 in His-LSP1 and SUMO1-overexpressed HEK 293T cells. In these cells, LSP1 was SUMOylated by SUMO1 and its SUMOylation was further enhanced by co-transfection of SUMO1 (Figure 6.1C). Taken together, the data suggest that LSP1 is SUMOylated by SUMO1.

6.2 LSP1 SUMOylation is aided by Ubc9 and deSUMOylation is mediated by SENP1

SUMOylation is catalyzed by a series of enzymes. SUMO is first activated by E1 enzymes (SAE1/SAE2), Ubc9 is the only E2 conjugating enzyme that precisely directs a SUMO molecule towards a specific target protein and E3 ligase enzymes (PIAS proteins) are important for the efficiency of SUMOylation [81, 96, 99, 232]. To study whether Ubc9 acts as the SUMO conjugating enzyme for LSP1, we co-expressed SUMO1 and His-LSP1 with or without Ubc9 in HEK 293T cells. Immunoblotting clearly showed that co-transfection of Ubc9 increased SUMOylation of His-LSP1 (Figure 6.2A, *left* panel) suggesting Ubc9 as the SUMO1 conjugating enzyme for LSP1.

SUMOylation is reversible and SUMO is removed from target proteins by specific SUMO proteases (SENPs) in an ATP-dependent manner. SENP1 is the protease responsible for the cleavage of SUMO1 [233]. To examine whether SENP1 can affect SUMOylation of LSP1, we co-expressed His-tagged LSP1 and SUMO1 in the presence or absence of SENP1 in HEK 293T cells. As shown in Figure 6.2A (*right panel*), the SUMOylated band of LSP1 was substantially reduced in cells transfected with SENP1 indicating SENP1 to be involved in the deSUMOylation of LSP1.

We observed very similar effect of Ubc9 and SENP1 upon their overexpression in EE2 endothelial cells (Figure 6.2B). These results provide clear evidence of Ubc9 and SENP1 being SUMO conjugase and protease, respectively, for LSP1 SUMOylation.

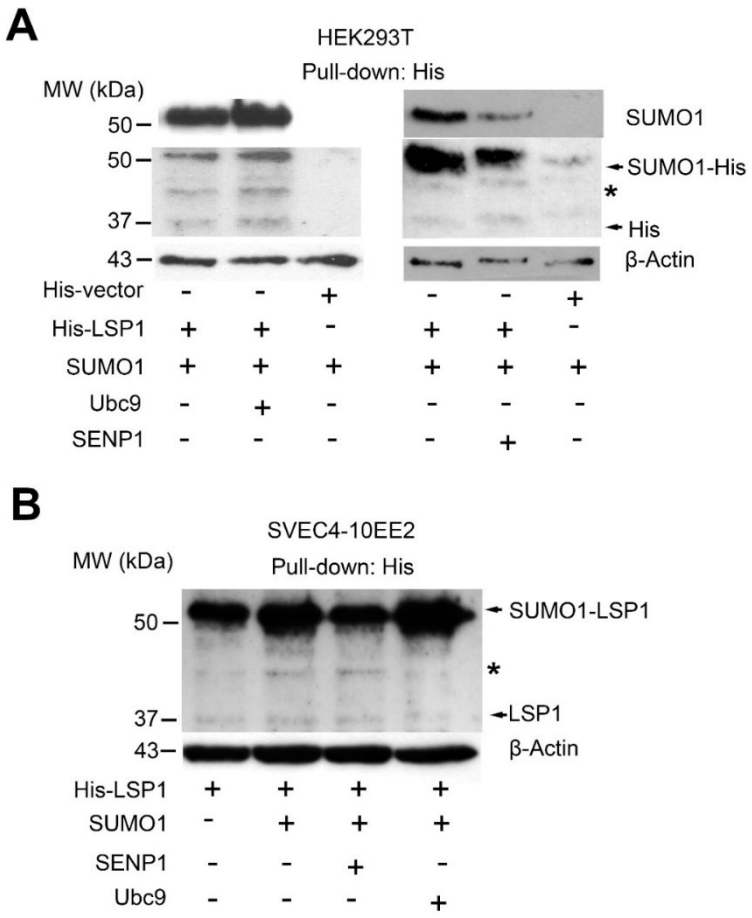

Figure 6.2: Ubc9 acts as SUMO conjugase and SENP1 as SUMO protease for LSP1. **(A)** Pulled-down His-tagged proteins from His-LSP1 and SUMO1-; His-LSP1, SUMO1 and Ubc9- or His-LSP1, SUMO1 and SENP1-overexpressed HEK 293T cells were immunoblotted with a monoclonal anti-SUMO1 or a polyclonal anti-His antibody. β-actin in the whole cell lysate was detected to ensure equal amount of total proteins was used for pulling down His-tagged proteins. Co-expression of Ubc9 (left) and SENP1 (right) substantially increased and decreased the LSP1-SUMO-1 conjugation, respectively (n = 3). Arrow indicates His or SUMO1-His conjugate and * indicates an unknown signal. **(B)** Pulled-down His-tagged proteins from His-LSP1 and SUMO1-; His-LSP1, SUMO1 and Ubc9-; His-LSP1, SUMO1 and SENP1- or His-LSP1-overexpressed EE2 cells were immunoblotted with anti-LSP1 rabbit serum. β-actin in the whole cell lysate was detected to ensure equal amount of total proteins was used for pulling down His-tagged proteins. Co-expression of SUMO1 and SENP1 significantly increased and reduced the LSP1-SUMO1 conjugation respectively (n = 3). Arrow indicates LSP1 or SUMO1-LSP1 conjugate and * indicates an unknown signal.

6.3 K270 and K318 are the primary sites of LSP1 SUMOylation

Having determined the posttranslational modification of LSP1 by SUMO1, we then examined the SUMO1 acceptor sites in LSP1. Mouse LSP1 does not have any generally known

consensus motif (ΨKxD/E) for SUMOylation. However, recent advancement of SUMOylation studies has revealed that this so-called consensus motif is not always necessary for SUMOylation. A number of proteins e.g., C-terminal-binding protein 2 (CtBP2), murine double minute 2 (Mdm2) and death-associated protein 6 (Daxx) that are lacking this motif can be SUMOylated [102-104] whereas even mutation of consensus motif cannot block SUMOylation of some other proteins such as proliferating cell nuclear antigen (PCNA) [234]. Moreover, a recent study has also identified some other SUMOylation motifs [235]. Therefore, in this study, we performed bioinformatic screening using SUMOplot™ (http://www.abgent.com/sumoplot), as described previously [88, 89, 236], to identify the most likely SUMO acceptor sites in LSP1. This screening revealed that K318 is the sole high probability SUMOylation site in the mouse LSP1 whereas in human LSP1, K279 and K327 are the high probability SUMOylation sites (Table 6.1).

Thus, in addition to K318, we considered K270, the well conserved murine analog of K279 of human LSP1 as the potential SUMOylation sites for mouse LSP1. Therefore, we performed site-directed mutagenesis of 270K→A (alanine), designated as His-LSP1^{K270A} and 318K→A, designated as His-LSP1^{K318A} in His-tagged LSP1 plasmids using PCR. Additionally, we randomly mutated K321 (321K→A, designated as His-LSP1^{K321A}) in mouse LSP1 to ensure that the mutation itself does not affect LSP1 expression. Expression of these mutants in HEK 293T cells led to the identification of K270 and K318 as the primary SUMOylation sites for mouse LSP1 (Figure 6.3).

Table 6.1: The high probability SUMO1 acceptor sites in mouse and human LSP1 as predicted by SUMOplot™

No.	Species	Position	Group	Score
1	Mouse	K318	VATGH **G<u>K</u>YE** KVLVD	0.67
	Human	K327	VATGH **G<u>K</u>YE** KVLVE	0.67
2	Mouse	K270	QSQSA **S<u>K</u>TP** SCQDI	---
	Human	K279	QAQSA **A<u>K</u>TP** SCKDI	0.69

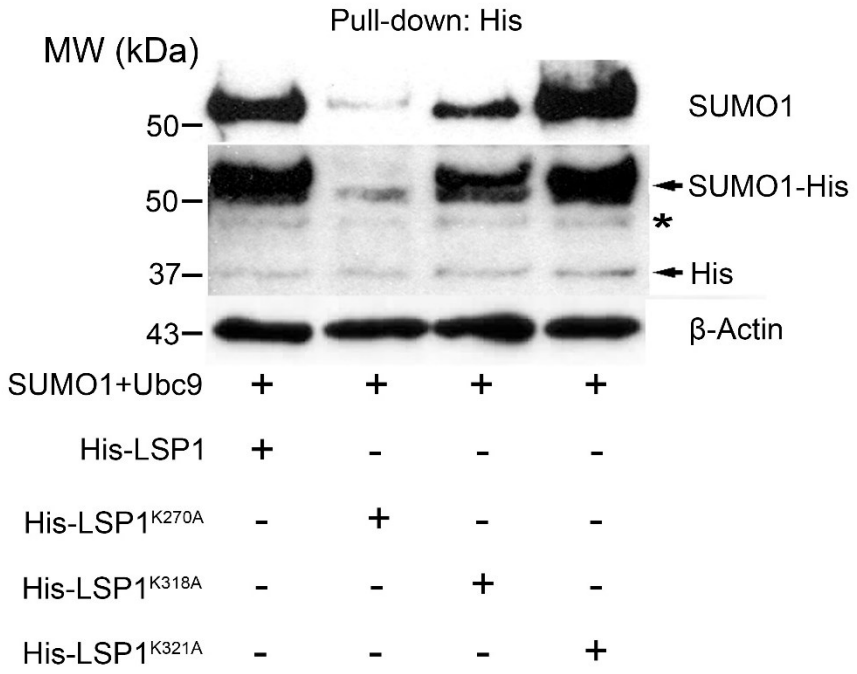

Figure 6.3: Lysine residues 270 and 318 are the primary sites of LSP1 SUMOylation. Pulled-down His-tagged proteins from SUMO1, Ubc9 and His-LSP1- or His-LSP1^{K270A}- or His-LSP1^{K318A}- or His-LSP1^{K321A}-co-expressed HEK 293T cells were immunoblotted with a monoclonal anti-SUMO1 or a polyclonal anti-His antibody. β-actin in the whole cell lysate was detected to ensure equal amount of total proteins was used for pulling down His-tagged proteins (n = 3). Arrow indicates His or SUMO1-His conjugate and * indicates an unknown signal.

6.4 DeSUMOylation affects LSP1 stability

SUMOylation is reported to affect a number of functions of the modified proteins; most notable of them are protein stability, nuclear localization of proteins, nuclear-cytosolic transport, and regulation of transcriptional activity [237-239]. At 24 hours of transfection, His-tagged protein band at 52 kDa was remarkably reduced in His-LSP1^{K270A} and His-LSP1^{K318A} transfected or His-LSP1 and SENP1 cotransfected HEK 293T cells compared to His-LSP1 transfected cells. Intriguingly, there was no corresponding increase in the 37 kDa His-tagged protein band in these cells (Figure 6.3) indicating the fact that deSUMOylation of LSP1 either by mutation of SUMOylation sites or by the action of SENP1 resulted in reduced steady-state level of LSP1. To this end, HEK 293T cells were transfected with equal amount of His-LSP1 or His-LSP1^{K270A} plasmids. Indeed, His-LSP1^{K270A} transfected cells yielded significantly lower level of LSP1 protein in total cell lysates compared to His-LSP1 transfected cells over a series of plasmid concentrations (Figure 6.4A). This observation indicates that reduction in SUMOyation decreases

steady-state protein level of LSP1. Steady-state protein level is altered because of the changes in either protein synthesis or protein degradation [240].

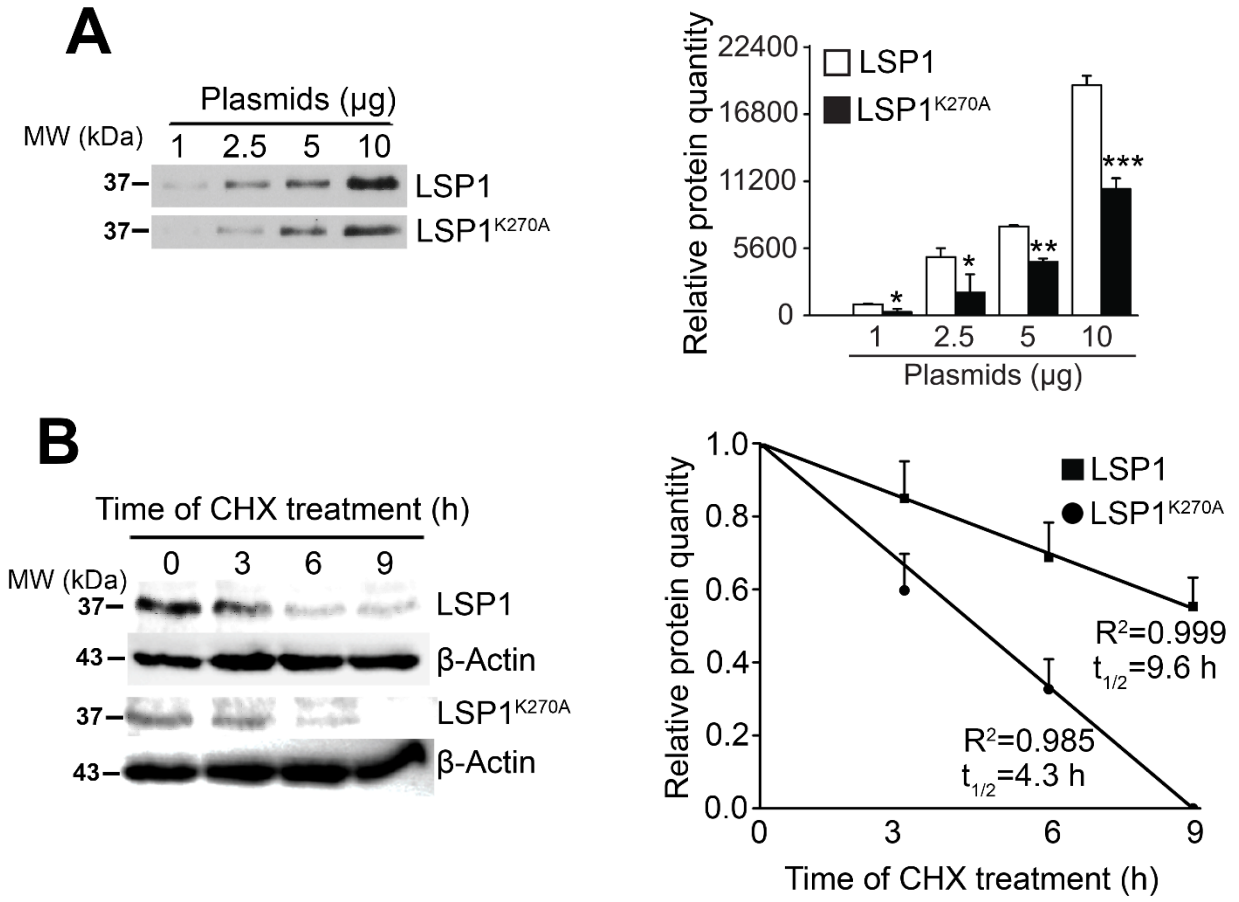

Figure 6.4: DeSUMOylated LSP1 has reduced protein levels and stability. **(A)** HEK 293T cells were transfected with a range (1-10 µg) of His-LSP1 or His-LSP1^{K270A} plasmid quantity. Twelve hours after transfection, cells were lysed and proteins were immunoblotted with a polyclonal anti-LSP1 antibody (left). Blots were analyzed by densitometry; results (means ± SD; n = 3) of three independent experiments were plotted as arbitrary unit (right). * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) from WT LSP1. **(B)** Twelve hours after His-LSP1 or His-LSP1^{K270A} transfection, HEK 293T cells were treated with 100 mg/ml cycloheximide (CHX) for the indicated time periods. Cells were lysed and proteins were immunoblotted using anti-LSP1 antibody (relative to β-actin; left). Blots were analyzed by densitometry; results (means ± SD; n = 3) of three independent experiments were plotted as arbitrary unit (relative to initial time points) and half-lives ($t_{1/2}$) were calculated by linear regression analysis of the fitted curves (right).

We then explored the role of SUMOylation on LSP1 stability. WT His-LSP1 or His-LSP1^{K270A} plasmids were transfected into HEK 293T cells. After 12 hours, cells were treated with cycloheximide for different time points to block any further protein synthesis. This is a commonly

used technique to follow the degradation kinetics of a particular protein over a certain period of time [81, 88, 89, 196]. Cycloheximide treated cells were lysed at indicated time points and immunoblotted with anti-LSP1 antibody. Densitometric quantification of the detected bands revealed that the mutant LSP1 was degraded at a much faster rate than its WT counterpart. Half-lives of WT and mutant LSP1 were calculated from the fitted curves obtained by linear regression analysis of these densitometric data. Half-life of the mutant LSP1 was only 4.3 hours as opposed to the half-life of the WT LSP1 (9.6 hours) (Figure 6.4B). Moreover, to determine whether mutation of SUMOylation site affected the expression of LSP1, we measured the mRNA level in His-LSP1 or His-LSP1^{K270A} plasmid transfected HEK 293T cells. Levels of LSP1 mRNA expression did not differ in WT or mutant LSP1 plasmid transfected HEK 293T cells at 12 hours after transfection (Figure 6.5A). These results indicate that the deSUMOylation-induced reduction of steady-state LSP1 level was related to increased degradation of LSP1 proteins.

6.5 Inhibition of SUMOylation accelerates ubiquitination-mediated proteasomal degradation of endothelial LSP1

Proteasomal degradation is one of the major pathways for the degradation of proteins in eukaryotic cells. To determine whether the deSUMOylated LSP1 is undergoing proteasomal degradation, HEK 293T cells were transfected with His-LSP1, His-LSP1^{K270A} or co-transfected with His-LSP1 and SENP1 followed by treatment with or without proteasome inhibitor MG132 (10 μM) for 12 hours. Immunoblotting of pulled-down His-tagged proteins with anti-LSP1 rabbit serum clearly shows that in the absence of MG132 treatment, LSP1 degrades at a much faster rate when SUMOylation is inhibited by mutation of the SUMOylation site or by co-expressing SENP1. MG132 treatment recovered both WT and mutant LSP1 from proteasomal degradation, however, the recovery in the mutant group was more dramatic (Figure 6.5B).

In eukaryotic cells, polyubiquitination often directs a protein to proteasomal degradation. To investigate the involvement of the ubiquitin-proteasome pathway in the accelerated degradation of deSUMOylated LSP1, HEK 293T cells were co-transfected with His-LSP1 or His-LSP1^{K270A} and HA-tagged ubiquitin followed by treatment with or without proteasome inhibitor MG132 (10μM) for 12 hours. Immunoblotting of purified His-tagged proteins with anti-ubiquitin antibody revealed that without MG132 treatment, ubiquitination of the mutant LSP1 was much higher relative to WT LSP1 (Figure 6.5C). With MG132 treatment, very high level of ubiquitination was observed in mutant LSP1 compared to that of without MG132 treatment (Figure 6.5C). For the

WT LSP1, MG132 treatment caused a slight increase in ubiquitination compared to without MG132 treatment. Taken together, these results indicate that SUMOylation protetcts LSP1 from proteasomal degradation which is consistent with a number of previous reports [81, 197, 240]. Immunoblotting of His-tagged proteins pulled-down from His-LSP1^{K270A} and His-LSP1^{K318A} transfected or His-LSP1 and SENP1 cotransfected HEK 293T cells in the presence or absence of proteasome inhibitor MG132 with anti-LSP1 rabbit serum revealed that rapid degradation of deSUMOylated LSP1 can be inhibited by proteasome inhibitor MG132.

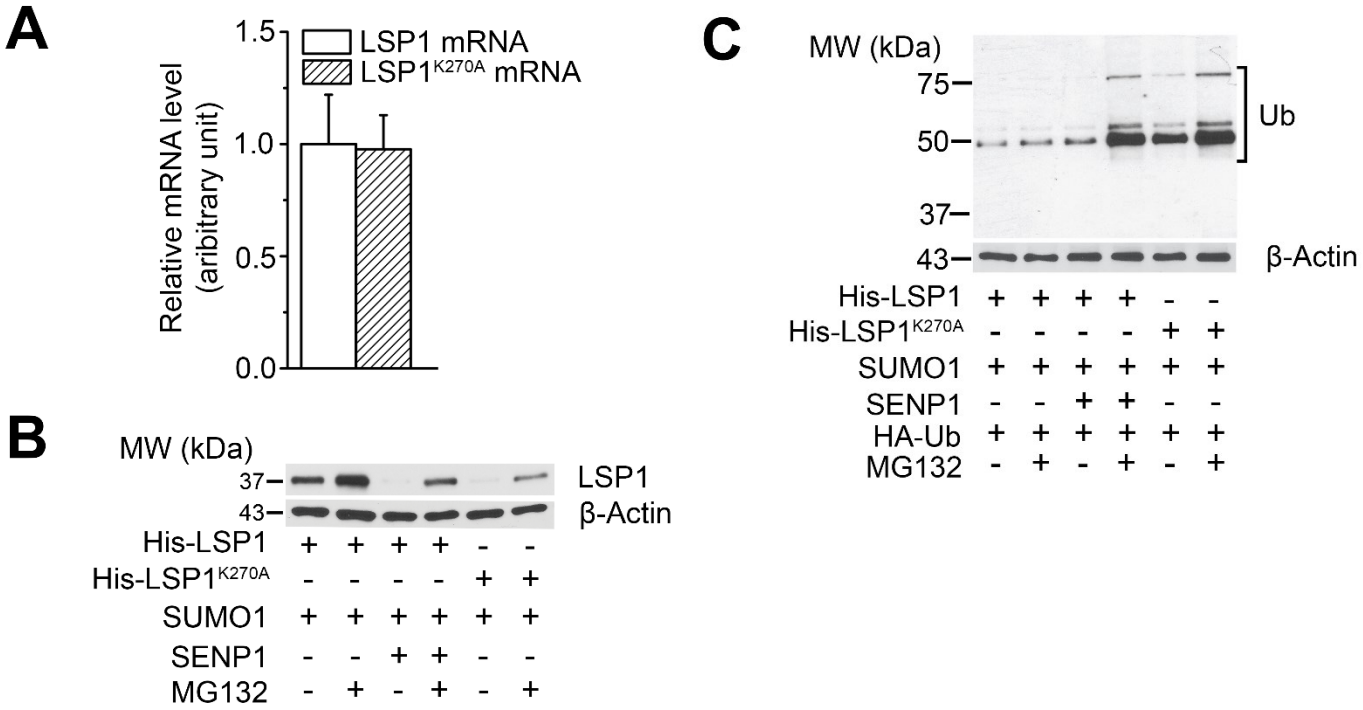

Figure 6.5: DeSUMOylation leads to polyubiquitination and subsequent proteasomal degradation of LSP1. (A) Means ± SD of mRNA levels (n = 3) encoding LSP1 in His-LSP1- or His-LSP1^{K270A}-transfected HEK 293T cells. (B) HEK 293T cells were transfected with His-LSP1 and SUMO1; His-LSP1, SUMO1 and SENP1 or His-LSP1^{K270A} and SUMO1 plasmids. After 12 h, cells were treated with or without 10 μM MG132 for 12 h and lysed and proteins were immunoblotted with anti-LSP1 antibody (relative to β-actin; n = 3). (C) HEK 293T cells were transfected with His-LSP1, SUMO1 and HA-Ub; His-LSP1, SUMO1, SENP1 and HA-Ub or His-LSP1^{K270A}, SUMO1 and HA-Ub plasmids. After 12 h, cells were treated with or without μM MG132 for 12 h and lysed and proteins were immunoblotted with anti-ubiquitin antibody (relative to β-actin; n = 3).

Because ubiquitination is well known for directing the modified proteins to proteasomal degradation, we explored whether LSP1 is modified by ubiquitin in the presence or absence of proteasome inhibitor. Acceleration of ubiquitination pathway by overexpressing ubiquitin led to the identification of LSP1 ubiquitination which was increased in the presence of MG132. Moreover, at the earlier stages of expression, deSUMOylated LSP1 shows high level of ubiquitination which increases dramatically at later stages but visible only in the presence of MG132 (Figure 6.5C). This observation is in agreement with some previous reports showing that the attachment of SUMO-1 to proteins prevents their ubiquitination and subsequent proteasomal degradation either by blocking the ubiquitination site [241] or by making the ubiquitin binding site inaccessible to the ubiquitin machinery [240, 242]. For LSP1, the latter is more appropriate since SUMO and ubiquitin do not compete for the same lysine residue. These results also clarify that the stability of LSP1 is regulated by the interplay between its SUMOylation and ubiquitination.

6.6 SUMOylation deficiency impairs nucleus to extra-nuclear translocation of LSP1

SUMOylation is reported, in numerous occasions, to be associated with compartmentalization [88, 184] and or translocation [239, 243] of many proteins. LSP1 in endothelial cells is distributed unequally in different compartments: nucleus being the major one [136]. Endothelial activation by TNF-α treatment causes translocation of some nuclear LSP1 into extranuclear compartments [37]. We also explored the possibility of SUMOyolation being a regulator of these LSP1 compartmentalization and translocation.

We overexpressed WT LSP1 with or without SENP1 or mutant LSP1 in EE2 cells and treated them with or without TNF-α. Immunoblotting followed by sub-cellular fractionation of these cells shows the evidence of LSP1 SUMOylation in all the sub-cellular compartments analyzed (Figure 6.6A) although the majority of SUMOylated LSP1 in unstimulated endothelial cells remain in the nucleus. Interestingly, the expression of the mutant LSP1 was almost exclusively in the nucleus. Moreover, TNF-α-induced translocation of the mutant LSP1 from nuclear to extra-nuclear compartments was minimal (Figure 6.6A-C). Coexpression of SENP1 also appeared to inhibit this translocation of the WT LSP1. These observations suggest the involvement of SUMOylation in nucleus to extra-nuclear translocation of LSP1.

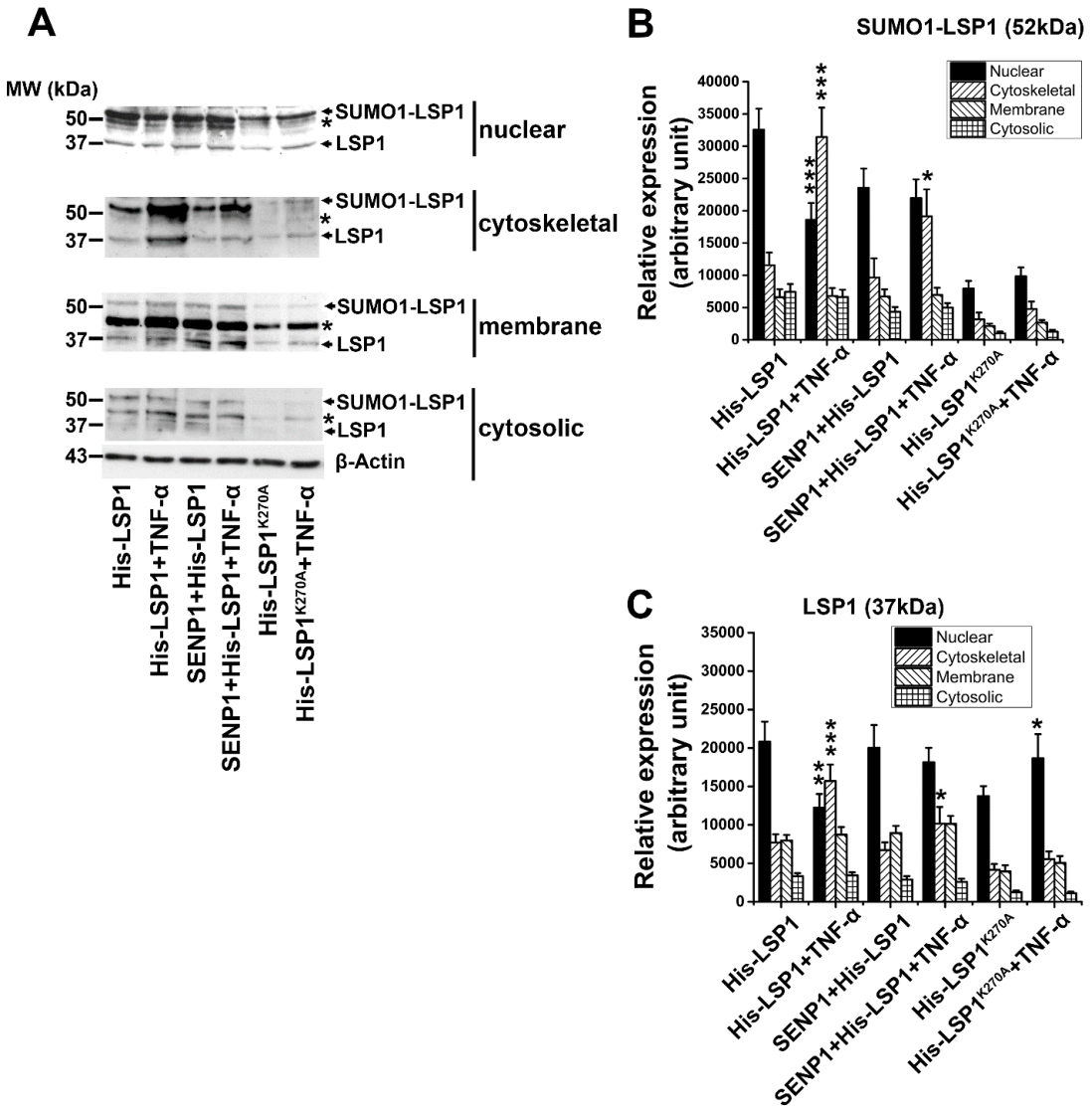

Figure 6.6: SUMOylation deficiency impairs the nucleus-to-extranuclear translocation of LSP1. (A) EE2 cells were transfected with His-LSP1 and SENP1, His-LSP1 or His-LSP1^{K270A} plasmids. After 12 h, cells were treated with or without TNF-α for 4 h. Six million cells from each group were then subjected to subcellular fractionation and four different fractions (in the sequential order of cytosolic, membrane, nuclear and cytoskeletal) were collected. Proteins from all four fractions were immunoblotted with anti-LSP1 rabbit serum (relative to β-actin in the cytosolic fraction; n = 3). Arrow indicates LSP1 or SUMO1-LSP1 conjugate and * indicates an unknown signal. (B) Densitometric analysis of SUMO1-LSP1 conjugate bands (52 kDa) and (C) densitometric analysis of LSP1 bands (37 kDa) in four subcellular fractions before and after TNF-α treatment. Data are presented as means ± SD (n = 3) of three independent experiments. * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) compared to without TNF-α treatment.

6.7 Summary

The present study is the first to reveal the posttranslational modification of LSP1 by SUMO1. During SUMOylation and deSUMOylation of LSP1, Ubc9 acts as SUMO conjugating enzyme whereas SENP1 acts as a SUMO protease. Using bioinformatics screening and site-directed mutagenesis, we identified two lysine residues (K270 and K318) in the C-terminus of LSP1 as the primary SUMOylation sites of LSP1. SUMOylation of LSP1 increases its stability by inhibiting ubiquitination-mediated rapid proteasomal degradation. This could be the reason for low relative abundance of the deSUMOylated LSP1 in endothelial cells at any given time. Additionally, SUMOylation is also important for the translocation of LSP1 from nucleus to extranuclear compartments. Whether posttranslational modification of LSP1 by SUMOylation represents a novel regulator of microvascular permeability and leukocyte infiltration in inflammatory diseases remains to be determined.

7.0 GENERAL DISCUSSION

7.1 Endothelial cell-expressed LSP1 regulates endothelial functions

Despite being nearly identical, endothelial cell- and leukocyte-expressed LSP1 differ dramatically in their functions and subcellular distribution [37, 136, 137]. In leukocytes, LSP1 has been shown to either enhance or decrease adhesion, emigration, polarization, chemotaxis, F-actin polymerization and cytoskeletal remodeling [146-148, 208]. In endothelial cells, LSP1 is shown to play an important role in neutrophil transmigration and endothelial permeability. However, how endothelial cell-expressed LSP1 regulates these functions is not well understood. Moreover, whether endothelial cell-expressed LSP1 affects other steps of leukocyte recruitment and other endothelial cells functions have not been addressed. To study the cell-specific role of a protein, the use of endothelial cells and neutrophils from the respective gene knock-out and their WT counterpart is considered ideal. However, in this thesis, we used LSP1-silenced endothelial cells (EE2 cells) because of the difficulty in isolating adequate primary endothelial cells from mouse pups.

7.1.1 Endothelial cell-expressed LSP1 in neutrophil adhesion and transmigration

Existing literature, albeit sometimes contradictory, supports the regulatory role of neutrophil-expressed LSP1 on adhesion as well as motility phenotype of neutrophils *in vitro* [146-149, 208]. Using intravital microscopy, it was also reported that LSP1-deficiency in general reduces neutrophil adhesion [136]. To address these discrepancies, we measured the adhesion of WT as well as KO neutrophils onto WT or KO endothelial cells *in vitro*. We observed that both neutrophil- and endothelial cell-expressed LSP1 are important for neutrophil adhesion on endothelial monolayers. However, endothelial cell-expressed LSP1 contributes more to this function as depicted in Figure 4.3A. Using WT and LSP1 silenced EE2 cells and WT or LSP1-deficient neutrophils, we have also studied cell-specific role of LSP1 in neutrophil transmigration across endothelial cell monolayers. Unlike adhesion, neutrophil transendothelial migration is regulated by endothelial cell- but not neutrophil-expressed LSP1 (Figure 4.3B).

In leukocytes, chemoattractant stimulation results in p38 MAPK-mediated upregulation of Mac-1 and RhoA-mediated LFA-1 activation [216, 244]. These adhesion molecules are crucial for neutrophil adhesion. In endothelial cells, however, chemoattractant treatment as well as

neutrophil adhesion can activate p38 MAPK with different consequences [210, 245, 246]; Figure 3.2, Figure 3.3 and Figure 3.5). LSP1 is a well-known signaling molecule downstream of both p38 MAPK and PKC pathways [153, 208]. Neutrophil-expressed LSP1 is efficiently activated by these kinases when neutrophils are treated with chemokines whereas LSP1 activation in endothelial cells requires p38 MAPK activation specifically following neutrophil adhesion (Figure 3.2 and Figure 3.3). Being downstream of these kinases, it is likely that LSP1 affects neutrophil adhesion by modulating these signaling pathways. Neutrophil-expressed LSP1 is likely to be involved in earlier stages of adhesion since it is activated directly upon chemokine exposure. On the other hand, endothelial cell-expressed LSP1 is probably involved in the later stages of adhesion (firm adhesion) since its activation requires neutrophil adhesion onto endothelial cells. Delayed activation may underscore a more important role of endothelial cell-expressed LSP1 in the subsequent steps of leukocyte recruitment.

Neutrophil transendothelial migration involves complex interactions between neutrophil- and endothelial cell-expressed adhesion molecules and subsequent cellular signaling [40]. Efficient transmigration requires cytoskeletal remodeling in neutrophils for extending pseudopods in search for a suitable place and in endothelial cells for opening up cell-cell junctions or the formation of transmigratory dome-like structures [14]. LSP1 interacts with F-actin and remodels cellular cytoskeleton. It regulates transcellular neutrophil migration via aiding in the formation of transmigratory domes in endothelial cells [37]. However, in most vascular beds, transcellular neutrophil migration is a less prevalent event [247, 248]. A more prominent role of endothelial cells-expressed LSP1 in neutrophil transmigration suggests its involvement also in paracellular neutrophil transmigration. Paracellular transmigration is primarily regulated by junctional adhesion molecules such as PECAM-1 and intracellular signaling events that lead to cytoskeletal rearrangement to open up the endothelial cell-cell contacts [14, 40]. Here, we have demonstrated that endothelial cell-expressed LSP1 regulates endothelial PECAM-1 expression by transcriptional regulation of the transcription factor GATA-2 (Figure 4.5 and Figure 4.6). So, reduced expression of PECAM-1 in LSP1-deficient endothelium surely affects paracellular migration of neutrophils. Interaction of LSP1 with moesin (Figure 5.1), a cytoskeleton binding protein involved in cytoskeleton remodeling, results in endothelial cell shape changes favouring its paracellular or transcellular migration. Thus, activated LSP1 significantly contributes to

endothelial cell shape changes. Taken together, it is clear that LSP1 regulates neutrophil adhesion and transmigration through several distinct mechanisms.

7.1.2 Endothelial cell-expressed LSP1 in endothelial cell permeability and migration

Endothelial cell permeability is differentially regulated by different stimuli [3]. Agents that are capable of regulating cell contraction (shape) such as histamine and bradykinin, increase endothelial permeability by opening up the cell-cell contact. On the contrary, damages to the endothelium by oxidants released from activated neutrophils or by thermal injury cause endothelial cell death and detachment. Neutrophil transmigration-induced hypermeability was found to be higher in chimeric mice with WT neutrophils but selectively deficient in endothelial cell-expressed LSP1 compared to mice with LSP1-deficient neutrophils and WT endothelial cells when calculated as permeability changes per transmigrated neutrophil [37]. Depletion of neutrophils in both groups brought the permeability changes to identical level indicating an important but yet unknown interaction between neutrophils and endothelial cells (not neutrophil generated reactive oxygen species) is central to this permeability changes [37]. Histamine-induced endothelial permeability changes, however, have an opposite trend. Histamine-mediated hypermeability is mediated by endothelial cell retraction [3, 4]. This is found to be higher in WT mice compared to their LSP1-deficient counterpart [136]. In our study, we used ICAM-1 cross-linking in TNF-α-treated endothelial monolayers or neutrophil migration in a MIP-2 gradient across endothelial monolayers before and after LSP1, moesin or GATA-2 silencing. We found striking differences in endothelial permeability changes between the neutrophil containing and lacking groups (Figure 5.4).

Neutrophil adhesion or transmigration is often associated with endothelial damage resulting in dysregulation of endothelial barrier function [249, 250]. Although neutrophil-endothelial cell interactions initiate a large number of endothelial signaling pathways, vascular hypermeability mediated by activated neutrophils is historically credited to the disruption of endothelial barriers due to direct endothelial injury by neutrophil-derived reactive oxygen species and granular products [250]. PECAM-1 has been known for a long time to be linked to the regulation of endothelial barrier functions and vascular permeability changes [221, 251-253]. It is also known that PECAM-1 homophilic interaction but not PECAM-1-mediated endothelial signaling is crucial for maintaining endothelial permeability [253]. Reduced PECAM-1 expression in LSP1-deficient endothelial cells could explain the vulnerability of LSP1-deficient endothelium

to neutrophil transmigration-mediated hypermeability. In contrast, in the absence of neutrophils, ICAM-1 clustering-mediated endothelial hypermeability is significantly higher in WT or GATA-2-silenced endothelial cells compared to LSP1- or moesin-silenced endothelial cells. During neutrophil-endothelial cell interactions, ICAM-1 engagement initiates many signaling pathways in endothelial cells including calcium signaling, activation of Rho kinase, PKC and p38 MAPK pathways [4, 7, 43, 254]. These pathways ultimately regulate endothelial cell retraction by remodeling intracellular cytoskeleton.

Taken together, our data confirms that in the absence of neutrophil adhesion or transmigration, endothelial cell-expressed LSP1 regulates endothelial permeability mainly by modulating endothelial cell retraction whereas in the presence of activated neutrophils, endothelial cell-expressed LSP1 regulates endothelial permeability mainly by affecting endothelial barrier function. More specifically, it reduces PECAM-1 homophilic interaction in the endothelial monolayer.

7.2 Is nuclear localization of endothelial cell-expressed LSP1 relevant to its function(s)?

Endothelial cell contraction is crucial for paracellular migration and endothelial hypermeability, formation of docking structure that encapsulate transcellularly migrating neutrophils and endothelial cell migration requires rapid cytoskeletal remodeling. Upon activation, endothelial cell-expressed LSP1 per se or its interaction with moesin could regulate endothelial cell actin dynamics. It was reported that in TNF-α-treated endothelial cells, LSP1 migrates from nucleus to cytoskeletal compartment [37]. Based on this observation, Petri and colleagues [37] speculated that most of this cytoskeleton remodeling protein is stored in the nucleus away from cytoskeleton, like MK2 [255]. This is because LSP1 abundance could promote the formation of hair-like projections and adversely affect motility of other cell types [157, 158]. The exact reason for storing proteins in the nucleus is unknown. However, nuclear localization can serve as a temporary storage site for otherwise a very active protein or it may also imply that a particular protein has some function(s) in the nucleus.

In this thesis, we found that both endothelial-specific PECAM-1 and GATA-2 were downregulated in LSP1 deficiency or upon LSP1 silencing whereas in LSP1-deficient neutrophils, PECAM-1 expression was unchanged. In light of the strategic nuclear localization of endothelial

LSP1 [136] in contrast to its cytosolic presence in neutrophils [146], it is intriguing to consider the role of endothelial-cell specificity of GATA-2 function [76, 216] in fostering cell-specific transcriptional regulation of PECAM-1 expression. Although more studies are needed to establish the exact nuclear function of endothelial cell-expressed LSP1, it can be concluded that nuclear LSP1 is not a mere stored inactive form of LSP1. Rather, it actively regulates the transcription of PECAM-1 by modulating GATA-2 expression in endothelial cells. There is no known DNA binding motif in LSP1. Therefore, it is difficult to speculate how LSP1 regulates GATA-2 expression in endothelial cells. However, nuclear localization of endothelial cell-expressed LSP1 and its ability to interact with a variety of signaling molecules tempted us to assume that LSP1 may possibly regulate the expression of GATA-2 by 1) facilitating the activation of nuclear factor necessary for GATA-2 transcription, 2) binding to GATA-2 transcriptional repressor(s) thus making them unavailable, 3) making the DNA accessible to DNA polymerase via interacting with histone or even by 4) acting as a coactivator in GATA-2 transcription. Further research is necessary to examine which of these proposed mechanisms is operational in endothelial cells.

7.3 Endothelial cell-expressed LSP1 functions require ICAM-1 clustering-mediated phosphorylation

LSP1 phosphorylation is considered to be functionally important for LSP1 activation which is evident from the fact that phosphorylated LSP1 is able to bind F-actin in lamellipodia of activated neutrophils while non-phosphorylated LSP1 fails to do so [208]. F-actin rich projections, cell motility and LSP1 were shown to be linked together which is important in health and disease [37, 141, 158]. LSP1 also plays an important role in cytoskeletal remodeling and motility of endothelial cells ([37]; Figure 4.1). Almost all of the functions of endothelial cell-expressed LSP1 reported so far require endothelial shape change. LSP1 is important in transcellular neutrophil migration where it is necessary for the endothelial cells to form the docking structures via extending projections to the side of the neutrophils [37]. It also facilitates endothelial permeability increases in response to agents that increase endothelial permeability by contracting endothelial cells ([136]; Figure 5.4). In our studies, we have observed that ICAM-1 engagement-mediated permeability increase requires the interaction between LSP1 and moesin that does not exist in the absence of ICAM-1 clustering (Figure 5.1B). In leukocytes, LSP1 is phosphorylated very quickly when the cells are stimulated by soluble chemoattractants such as fMLP and chemokines [143,

153, 208]. On the contrary, ICAM-1-mediated neutrophil adhesion is essential for LSP1 phosphorylation in endothelial cells (Figure 3.2, Figure 3.4 & Figure 3.5). The fact that ICAM-1 engagement is required for both LSP1 phosphorylation and LSP1-moesin interaction related endothelial permeability changes indicates that LSP1 phosphorylation plays a key role in regulating cytoskeletal remodeling and contraction of endothelial cells. These results also provide the notion that LSP1-depndent endothelial cell motility or migration also relies on the phosphorylation of endothelial cell-expressed LSP1. So, LSP1 regulated endothelial functions are dependent on the phosphorylation of endothelial cell-expressed LSP1 which, in fact, relies mainly on ICAM-1 engagement.

7.4 SUMOylation help maintain endothelial cell-expressed LSP1 functions

In spite of having a number of indications of probable post-translational modifications of endothelial cell-expressed LSP1 such as discrepancy in molecular weight (predicted *versus* presented in SDS-PAGE is 37 kDa *versus* 52 kDa; [130, 141, 143, 182]), predominant nuclear distribution [136] of endothelial LSP1 and its translocation from nucleus to extranuclear compartments upon TNF-α stimulation [37]; nothing is really known about any such modifications. The present work addresses this issue from the endothelial cell standpoint of phosphorylation of endothelial cell-expressed LSP1 (Chapter 03). Here, in addition to the well-known 52-kDa form of LSP1, we have also detected a 37-kDa form of LSP1 in murine endothelial cells (Figure 6.1). Using recombinant LSP1, Ubc9, SENP1 and SUMO1 expression plasmids, we have successfully demonstrated that the abundant 52-kDa form of LSP1 was modified by SUMO1. For a SUMOylated protein, relative abundance of the SUMOylated fraction is usually quite low although the opposite result is also available [83]. This relative abundance may also vary depending on the cell lysis procedure and the type of lysis buffer being used [83]. Bioinformatic screening using SUMOplot™, as described previously [88, 89, 236], followed by site-directed mutation and immunoblotting, we successfully identified lysine 270 and 318 to be the primary SUMOylation sites for murine endothelial cell-expressed LSP1.

SUMOylation is reported to affect a number of functions of the modified proteins; most notable of them are protein stability, nuclear-cytosolic transport, and the regulation of transcriptional activity [237-239]. We observed that deSUMOylation of LSP1 either by mutation

of SUMOylation sites or by the action of SENP1 resulted in reduced steady-state levels of LSP1 even though mRNA level was identical in WT LSP1- or mutant LSP1^{K270A}-transfected HEK 293T cells. Since reduced steady-state levels of LSP1 can be due to reduced protein synthesis or protein degradation [240], we next explored degradation kinetics of WT LSP1 and mutant LSP1^{K270A} using cycloheximide chase assay. This assay clearly showed that the degradation of LSP1^{K270A} mutant was much faster (half-life = 4.3 h) than its WT counterpart (half-life = 9.6 h) and can be inhibited by proteasome inhibitor MG132. Because ubiquitination is well known for directing the modified proteins to proteasomal degradation, we explored whether LSP1 is modified by ubiquitin in the presence or absence of proteasome inhibitor. At the earlier stages of expression, deSUMOylated LSP1 shows high level of ubiquitination which was increased greatly at later stages but visible only in the presence of MG132. This observation is in agreement with some previous reports demonstrating that the attachment of SUMO1 to proteins protects them from ubiquitination and subsequent proteasomal degradation either by blocking the ubiquitination site [241] or by making the ubiquitin binding site inaccessible to the ubiquitin machinery [240, 242]. For LSP1, the latter is more appropriate since SUMO and ubiquitin do not compete for the same lysine residue. Taken together, these results indicate that SUMOylation protects LSP1 from ubiquitination-directed proteasomal degradation, a dynamic process that was observed for various proteins in a number of previous reports [81, 197, 240] and stability of LSP1 is regulated by the interplay between its SUMOylation and ubiquitination.

To address whether specialized subcellular distribution and translocation of endothelial LSP1 is related to SUMOylation, we performed subcellular fractionation analysis of LSP1-overexpressed EE2 cells after TNF-α treatment. Without TNF-α treatment, we were able to detect SUMOylated LSP1 in all four subcellular fractions we analyzed, however; the expression of LSP1^{K270A} was almost exclusively in the nucleus. TNF-α treatment was reported to result in a remarkable decrease in nuclear LSP1 with concomitant increase of LSP1 in other fractions predominantly in cytoskeleton of endothelial cells [37]. Using EE2 cells overexpressing WT LSP1, we observed similar translocation. Interestingly, this translocation was much lower in EE2 cells co-expressing LSP1 and SENP1 whereas in the mutant LSP1^{K270A}-overexpressed cells, there was no apparent LSP1 translocation. These observations clearly indicate the involvement of SUMOylation in nuclear export of LSP1 and are in agreement with many other reports showing SUMOylation to be a major player in nuclear export for a number of proteins [243, 256, 257].

In summary, our data provide strong evidence of SUMOylation of endothelial LSP1 which help maintain LSP1 stability and functions by protecting LSP1 from ubiquitination-mediated proteasomal degradation. SUMOylation also helps translocate LSP1 to the extranuclear compartments.

7.5 Conclusion

- Activation of either neutrophil or endothelial cell is sufficient to support neutrophil adhesion.
- ICAM-1-mediated neutrophil adhesion is necessary for the phosphorylation of endothelial cell-expressed LSP1.
- Phosphorylation of endothelial cell-expressed LSP1 is mediated by p38 MAPK pathway.
- LSP1 is an important regulator of many endothelial cell functions, such as endothelial permeability, endothelial cell motility, neutrophil adhesion onto endothelial cell and neutrophil transendothelial migration.
- ICAM-1 cross-linking mimics neutrophil adhesion and increases TNF-α-mediated endothelial permeability.
- ICAM-1 cross-linking-mediated endothelial hypermeability is dependent upon LSP1-moesin interaction and subsequent endothelial cell contraction whereas neutrophil-mediated direct endothelial injury is more important in neutrophil adhesion or transmigration-mediated endothelial hypermeability.
- LSP1 regulates many endothelial functions, e.g., endothelial cell motility and neutrophil adhesion/transmigration-mediated endothelial hypermeability by modulating GATA-2-dependent selective expression of PECAM-1 in endothelial cells and subsequently, the expression of $\alpha_6\beta_1$ integrins on the transmigratory neutrophils.
- LSP1 is post-translationally modified by SUMO1. SUMOylation help maintain LSP1 functions by preventing rapid proteasomal degradation of LSP1 and by helping nucleus-to-extranuclear transport of endothelial cell-expressed LSP1.

7.6 Clinical relevance

LSP1 plays important role in mammalian immunity in general and inflammation in particular. Overexpression of LSP1 leads to the formation of pseudopod-like projections and reduces motility of the LSP1 overexpressed cells. Overexpression of LSP1 in neutrophils reduces

their motility and causes neutrophil actin dysfunction (NAD 47/89) disease. Neutrophils from NAD 47/89 patients are susceptible to recurrent infections due to reduced motility of their LSP1-overexpressing neutrophils. On the other hand, normal level of LSP1 is necessary for leukocyte functions such as transmigration and chemotaxis. In endothelial cells, LSP1 is localized primarily in the nucleus and translocate into cytoskeleton upon inflammatory stimulus. Endothelial cell-expressed LSP1 is crucial for neutrophil transendothelial migration and extravascular chemotaxis. Both endothelial cell- and leukocyte-expressed LSP1 get activated by the p38 MAPK pathway, although by two different stimuli. Pharmacological inhibition of p38 MAPK pathway by using selecting inhibitors is an ongoing approach of discovering novel anti-inflammatory drugs. However, contrary to the manufacturer's claim, these inhibitors often inhibit other kinases at a relatively higher dose. Additionally, involvement of p38 MAPK pathway in many cellular processes such as cellular growth, differentiation and apoptosis making it less suitable as a drug target.

Since LSP1 is a downstream signalling molecule in the p38 MAPK pathway and silencing of LSP1 in endothelial cells *in vitro* reduces inflammatory phenotype in these cells, LSP1 may provide a more specific potential target for inflammatory diseases. However, the presence of LSP1 in many cell types pose a valid challenge in the process of LSP1 targeting potential drugs. In this regard, gene therapy utilizing Cre-Lox/P system can be very useful since it can precisely insert, delete or mutate LSP1 DNA sequences in specific target cell populations minimizing the effects of non-selective inhibition of LSP1 functions.

7.7 Future directions

7.7.1 Getting to the tissue and cell-specific role of LSP1 in inflammation

LSP1 certainly play an important role in inflammation in general and leukocyte recruitment in particular. Number of recruited leukocytes in the peritoneum [148] and knee joint [147] was found to be higher in the LSP1-deficient mice compared their wild-type counterpart. These studies were based on data from simple knock out and wild-type mice. Therefore, these reports do not provide any cell-specific role of LSP1. On the other hand, using bone marrow transplanted chimeric mice, it was specifically shown that endothelial cell-expressed LSP1 but not the neutrophil expressed LSP1 is important for neutrophil transendothelial migration and extravascular tissue chemotaxis in cremaster muscle [136, 258]. It is yet to be investigated whether

this discrepancy in the role of LSP1 in different models of leukocyte recruitment is due to differences in the role of cell-specific LSP1 or simply due to differences in the architecture of different tissues. Instead of using bone marrow transplanted chimeric mice, Cre-Lox recombination-mediated cell-specific deletion or insertion of LSP1 in specific cell types would clearly establish the cell-specific role of LSP1 *in vivo* in different models of inflammation in different tissues. Moreover, to rule out or in the involvement of tissue architecture in the reported differences, leukocyte recruitment studies are required to be carried out in tissues having blood vessels especially venules of different tissues such as mesenteric venules or pial venules.

7.7.2 Targeting LSP1 as a potential therapy

It is already known that overexpression of LSP1 impairs motile functions in leukocytes which is a major problem in NAD47/89 patients. However, the basal level of LSP1 expression is necessary for proper functioning of endothelial cells and leukocytes. So, generalized targeting of LSP1 will result in numerous unwanted effects. Instead, Cre-Lox recombination-mediated cell-specific targeting of LSP1 will dramatically reduce the untoward effects of generalized LSP1 knock-out. Gene therapy utilizing Cre-Lox/P system is being tested routinely *in vivo* and *in vitro* [259, 260]. Since Cre-Lox/P system can deliver the necessary DNA inserts for knocking out a gene in a specific cell type, it can easily be used to knock out LSP1 in the cell type(s) of interest. This kind of LSP1 targeting can provide a good option for the treatment of LSP1-related genetic disease, e.g., NAD47/89.

REFERENCES

1. Liu, J., et al., *Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes.* Cell, 1991. **66**(4): p. 807-815.
2. Leick, M., et al., *Leukocyte recruitment in inflammation: basic concepts and new mechanistic insights based on new models and microscopic imaging technologies.* Cell and Tissue Research, 2014. **355**(3): p. 647-656.
3. Kumar, V., A.K. Abbas, and J.C. Aster, *Robbins & Cotran Pathologic Basis of Disease*. 2014: Elsevier Health Sciences.
4. Sukriti, S., et al., *Mechanisms regulating endothelial permeability.* Pulmonary Circulation, 2014. **4**(4): p. 535-551.
5. Majno, G., S.M. Shea, and M. Leventhal, *Endothelial contraction induced by histamine-type mediators: an electron microscopic study.* The Journal of Cell Biology, 1969. **42**(3): p. 647-72.
6. Kang, Y.-H. and R. Williams, *Endotoxin-induced endothelial injury and subendothelial accumulation of fibronectin in rat aorta.* The Anatomical Record, 1991. **229**(1): p. 86-102.
7. Mehta, D. and A.B. Malik, *Signaling Mechanisms Regulating Endothelial Permeability.* Physiological Reviews, 2006. **86**(1): p. 279-367.
8. Carmeliet, P. and R.K. Jain, *Angiogenesis in cancer and other diseases.* Nature, 2000. **407**(6801): p. 249-257.
9. Schmidt, S., M. Moser, and M. Sperandio, *The molecular basis of leukocyte recruitment and its deficiencies.* Molecular Immunology, 2013. **55**(1): p. 49-58.
10. Woodfin, A., et al., *The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo.* Nature Immunology, 2011. **12**(8): p. 761-769.
11. Mathias, J.R., et al., *Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish.* Journal of Leukocyte Biology, 2006. **80**(6): p. 1281-1288.
12. Qadri, S., et al., *Endothelial-expressed leukocyte-specific protein 1 regulates intravascular and extravascular directionality of chemotaxing neutrophils (P5094).* The Journal of Immunology, 2013. **190**(Meeting Abstracts 1): p. 58.1.
13. Howard, T.H., J. Hartwig, and C. Cunningham, *Lymphocyte-Specific Protein 1 Expression in Eukaryotic Cells Reproduces the Morphologic and Motile Abnormality of NAD 47/89 Neutrophils.* Blood, 1998. **91**(12): p. 4786-4795.
14. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nature Reviews Immunology, 2013. **13**(3): p. 159-175.
15. McEver, R.P., *Selectins: lectins that initiate cell adhesion under flow.* Current Opinion in Cell Biology, 2002. **14**(5): p. 581-586.
16. Moser, M., et al., *The Tail of Integrins, Talin, and Kindlins.* Science, 2009. **324**(5929): p. 895-899.
17. Hogg, N., I. Patzak, and F. Willenbrock, *The insider's guide to leukocyte integrin signalling and function.* Nature Reviews Immunology, 2011. **11**(6): p. 416-426.
18. Hynes, R.O., *Integrins: Bidirectional, Allosteric Signaling Machines.* Cell, 2002. **110**(6): p. 673-687.
19. Herter, J. and A. Zarbock, *Integrin Regulation during Leukocyte Recruitment.* The Journal Immunology, 2013. **190**(9): p. 4451-4457.

20. Elks, P.M., et al., *Activation of hypoxia-inducible factor-1α (Hif-1α) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in a zebrafish inflammation model.* Blood, 2011. **118**(3): p. 712-722.
21. Buckley, C.D., et al., *Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration.* Journal of Leukocyte Biology, 2006. **79**(2): p. 303-311.
22. Hidalgo, A., et al., *Complete Identification of E-Selectin Ligands on Neutrophils Reveals Distinct Functions of PSGL-1, ESL-1, and CD44.* Immunity, 2007. **26**(4): p. 477-489.
23. Berlin, C., et al., *α4 integrins mediate lymphocyte attachment and rolling under physiologic flow.* Cell, 1995. **80**(3): p. 413-422.
24. Berlin-Rufenach, C., et al., *Lymphocyte Migration in Lymphocyte Function-associated Antigen (LFA)-1–deficient Mice.* The Journal of Experimental Medicine, 1999. **189**(9): p. 1467-1478.
25. Henderson, R.B., et al., *Rapid recruitment of inflammatory monocytes is independent of neutrophil migration.* Blood 2003. **102**(1): p. 328-335.
26. Summers, C., et al., *Neutrophil kinetics in health and disease.* Trends in Immunology, 2010. **31**(8): p. 318-324.
27. Ley, K., *Arrest Chemokines.* Microcirculation, 2003. **10**(3-4): p. 289-295.
28. Laudanna, C., et al., *Rapid leukocyte integrin activation by chemokines.* Immunological Reviews, 2002. **186**(1): p. 37-46.
29. Shamri, R., et al., *Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines.* Nature Immunology, 2005. **6**(5): p. 497-506.
30. Ginsberg, M.H., A. Partridge, and S.J. Shattil, *Integrin regulation.* Current Opinion in Cell Biology, 2005. **17**(5): p. 509-516.
31. Jenne, Craig N., et al., *Neutrophils Recruited to Sites of Infection Protect from Virus Challenge by Releasing Neutrophil Extracellular Traps.* Cell Host & Microbe, 2013. **13**(2): p. 169-180.
32. Phillipson, M., et al., *Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade.* The Journal of Experimental Medicine, 2006. **203**(12): p. 2569-2575.
33. Gorina, R., et al., *β2 Integrin–Mediated Crawling on Endothelial ICAM-1 and ICAM-2 Is a Prerequisite for Transcellular Neutrophil Diapedesis across the Inflamed Blood–Brain Barrier.* The Journal of Immunology, 2014. **192**(1): p. 324-337.
34. Phillipson, M., et al., *Vav1 Is Essential for Mechanotactic Crawling and Migration of Neutrophils out of the Inflamed Microvasculature.* The Journal of Immunology, 2009. **182**(11): p. 6870-6878.
35. Ley, K., et al., *Getting to the site of inflammation: the leukocyte adhesion cascade updated.* Nature Reviews Immunology, 2007. **7**(9): p. 678-689.
36. Phillipson, M. and P. Kubes, *The neutrophil in vascular inflammation.* Nature Medicine, 2011. **17**(11): p. 1381-1390.
37. Petri, B., et al., *Endothelial LSP1 is involved in endothelial dome formation, minimizing vascular permeability changes during neutrophil transmigration in vivo.* Blood, 2011. **117**(3): p. 942-52.
38. Reymond, N., et al., *DNAM-1 and PVR Regulate Monocyte Migration through Endothelial Junctions.* The Journal of Experimental Medicine, 2004. **199**(10): p. 1331-1341.

39. Burns, A.R., et al., *Neutrophil transendothelial migration is independent of tight junctions and occurs preferentially at tricellular corners.* The Journal of Immunology, 1997. **159**(6): p. 2893-903.
40. Muller, W.A., *Mechanisms of Leukocyte Transendothelial Migration.* Annual Review of Pathology: Mechanisms of Disease, 2011. **6**(1): p. 323-344.
41. Vestweber, D., *Relevance of endothelial junctions in leukocyte extravasation and vascular permeability.* Annals of the New York Academy of Sciences, 2012. **1257**(1): p. 184-192.
42. Muller, W.A., *Leukocyte–endothelial-cell interactions in leukocyte transmigration and the inflammatory response.* Trends in Immunology, 2003. **24**(6): p. 326-333.
43. Huang AJ, M.J., Bandak TM, Ratau MC, Hanser KR, Silverstein SC., *Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells.* The Journal of Cell Biology, 1993. **120**(6): p. 1371-1380.
44. Barreiro, O., et al., *Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes.* The Journal of Cell Biology, 2002. **157**(7): p. 1233-1245.
45. Carman, C.V. and T.A. Springer, *A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them.* The Journal of Cell Biology, 2004. **167**(2): p. 377-388.
46. Hallmann, R., et al., *Expression and Function of Laminins in the Embryonic and Mature Vasculature.* Physiological Reviews, 2005. **85**(3): p. 979-1000.
47. Wang, S., et al., *Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils.* The Journal of Experimental Medicine, 2006. **203**(6): p. 1519-1532.
48. Dangerfield, J., et al., *PECAM-1 (CD31) homophilic interaction up-regulates alpha6beta1 on transmigrated neutrophils in vivo and plays a functional role in the ability of alpha6 integrins to mediate leukocyte migration through the perivascular basement membrane.* The Journal of Experimental Medicine 2002. **196**(9): p. 1201-11.
49. Stefanidakis, M. and E. Koivunen, *Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and cancer progression.* Blood, 2006. **108**(5): p. 1441-1450.
50. Madri, J.A. and D. Graesser, *Cell Migration in the Immune System: the Evolving Inter-Related Roles of Adhesion Molecules and Proteinases.* Developmental Immunology, 2000. **7**(2-4): p. 103-116.
51. Foxman, E.F., J.J. Campbell, and E.C. Butcher, *Multistep Navigation and the Combinatorial Control of Leukocyte Chemotaxis.* The Journal of Cell Biology, 1997. **139**(5): p. 1349-1360.
52. Funamoto, S., et al., *Spatial and Temporal Regulation of 3-Phosphoinositides by PI 3-Kinase and PTEN Mediates Chemotaxis.* Cell, 2002. **109**(5): p. 611-623.
53. Heit, B., et al., *PTEN functions to 'prioritize' chemotactic cues and prevent 'distraction' in migrating neutrophils.* Nature Immunology, 2008. **9**(7): p. 743-752.
54. Gill, G., *SUMO and ubiquitin in the nucleus: different functions, similar mechanisms?* Genes & Development, 2004. **18**(17): p. 2046-2059.
55. Rousseau, S., et al., *p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells.* Oncogene, 1997. **15**(18): p. 2169-77.

56. Lamalice, L., F. Le Boeuf, and J. Huot, *Endothelial Cell Migration During Angiogenesis.* Circulation Research, 2007. **100**(6): p. 782-794.
57. Pinedo, H.M., et al., *Involvement of platelets in tumour angiogenesis?* The Lancet. **352**(9142): p. 1775-1777.
58. Seljelid, R., S. Jozefowski, and B. Sveinbjornsson, *Tumor stroma.* Anticancer research, 1999. **19**(6A): p. 4809-22.
59. Heymans, S., et al., *Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure.* Nature Medicine, 1999. **5**(10): p. 1135-1142.
60. O'Reilly, M.S., et al., *Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a lewis lung carcinoma.* Cell. **79**(2): p. 315-328.
61. O'Reilly, M.S., et al., *Endostatin: An Endogenous Inhibitor of Angiogenesis and Tumor Growth.* Cell. **88**(2): p. 277-285.
62. Schaper, W. and W.D. Ito, *Molecular Mechanisms of Coronary Collateral Vessel Growth.* Circulation Research, 1996. **79**(5): p. 911-919.
63. Buschmann, I. and W. Schaper, *The pathophysiology of the collateral circulation (arteriogenesis).* The Journal of Pathology, 2000. **190**(3): p. 338-342.
64. Huang, T.T., et al., *Sequential Modification of NEMO/IKKγ by SUMO-1 and Ubiquitin Mediates NF-κB Activation by Genotoxic Stress.* Cell, 2003. **115**(5): p. 565-576.
65. Mastrangelo, A., et al., *The Role of Posttranslational Protein Modifications in Rheumatological Diseases: Focus on Rheumatoid Arthritis.* Journal of Immunology Research, 2015. **2015**: p. 712490.
66. Marks, F., *Protein Phosphorylation*. 2008: Wiley.
67. Burnett, G. and E.P. Kennedy, *The enzymatic phosphorylation of proteins.* The Journal of Biological Chemistry, 1954. **211**(2): p. 969-80.
68. Fischer, E.H. and E.G. Krebs, *Conversion of phosphorylase b to phosphorylase a in muscle extracts.* The Journal of Biological Chemistry, 1955. **216**(1): p. 121-32.
69. Sutherland, E.W., Jr. and W.D. Wosilait, *Inactivation and activation of liver phosphorylase.* Nature, 1955. **175**(4447): p. 169-70.
70. Guan, K. and J. Dixon, *Protein tyrosine phosphatase activity of an essential virulence determinant in Yersinia.* Science, 1990. **249**(4968): p. 553-556.
71. Cohen, P., *The role of protein phosphorylation in human health and disease.* European Journal of Biochemistry, 2001. **268**(19): p. 5001-5010.
72. Shenoy, S.K., *Seven-Transmembrane Receptors and Ubiquitination.* Circulation Research, 2007. **100**(8): p. 1142-1154.
73. Hershko, A. and A. Ciechanover, *THE UBIQUITIN SYSTEM.* Annual Review of Biochemistry, 1998. **67**(1): p. 425-479.
74. Grabbe, C., K. Husnjak, and I. Dikic, *The spatial and temporal organization of ubiquitin networks.* Nature Reviews Molecular Cell Biology, 2011. **12**(5): p. 295-307.
75. Hasan, S.M.R., *Modulation of the TRPA1 and TRPV1 ion channels*, in *Department of Pharmacology,* 2014, The University of Cambridge Cambridge, England. p. 140.
76. d'Azzo, A., A. Bongiovanni, and T. Nastasi, *E3 Ubiquitin Ligases as Regulators of Membrane Protein Trafficking and Degradation.* Traffic, 2005. **6**(6): p. 429-441.
77. Pickart, C.M. and D. Fushman, *Polyubiquitin chains: polymeric protein signals.* Current Opinion in Chemical Biology, 2004. **8**(6): p. 610-616.

78. Hicke, L., *Protein regulation by monoubiquitin.* Nature Reviews Molecular Cell Biology, 2001. **2**(3): p. 195-201.
79. Salmena, L. and P.P. Pandolfi, *Changing venues for tumour suppression: balancing destruction and localization by monoubiquitylation.* Nature Reviews Cancer, 2007. **7**(6): p. 409-413.
80. Mukhopadhyay, D. and H. Riezman, *Proteasome-Independent Functions of Ubiquitin in Endocytosis and Signaling.* Science, 2007. **315**(5809): p. 201-205.
81. Xing, X., et al., *SUMOylation of AhR modulates its activity and stability through inhibiting its ubiquitination.* Journal of Cellular Physiology, 2012. **227**(12): p. 3812-9.
82. Zhao, J., *Sumoylation regulates diverse biological processes.* Cellular and Molecular Life Sciences, 2007. **64**(23): p. 3017-3033.
83. Mahajan, R., et al., *A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2.* Cell, 1997. **88**(1): p. 97-107.
84. Matunis MJ, C.E., Blobel G *A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex.* The Journal of Cell Biology, 1996. **135**(6): p. 1457-1470.
85. Wimmer, P., S. Schreiner, and T. Dobner, *Human Pathogens and the Host Cell SUMOylation System.* Journal of Virology, 2012. **86**(2): p. 642-654.
86. Sarge, K.D. and O.-K. Park-Sarge, *Sumoylation and human disease pathogenesis.* Trends in Biochemical Sciences, 2009. **34**(4): p. 200-205.
87. Wei, W., et al., *A stress-dependent SUMO4 sumoylation of its substrate proteins.* Biochemical and Biophysical Research Communications, 2008. **375**(3): p. 454-459.
88. Yang, F., et al., *Sumoylation is important for stability, subcellular localization, and transcriptional activity of SALL4, an essential stem cell transcription factor.* The Journal of Biological Chemistry, 2012. **287**(46): p. 38600-8.
89. de Cristofaro, T., et al., *Pax8 protein stability is controlled by sumoylation.* Journal of Molecular Endocrinology, 2009. **42**(1): p. 35-46.
90. Zhou, W., et al., *SUMOylation of HNF4alpha regulates protein stability and hepatocyte function.* Journal of Cell Science, 2012. **125**(Pt 15): p. 3630-5.
91. Kjenseth, A., et al., *The gap junction channel protein connexin 43 is covalently modified and regulated by SUMOylation.* The Journal of Biological Chemistry, 2012. **287**(19): p. 15851-61.
92. Dehennaut, V., et al., *DNA double-strand breaks lead to activation of hypermethylated in cancer 1 (HIC1) by SUMOylation to regulate DNA repair.* The Journal of Biological Chemistry, 2013. **288**(15): p. 10254-64.
93. Kang, J.S., et al., *The type I TGF-beta receptor is covalently modified and regulated by sumoylation.* Nature Cell Biology, 2008. **10**(6): p. 654-64.
94. Miyazono, K., Y. Kamiya, and K. Miyazawa, *SUMO amplifies TGF-beta signalling.* Nature Cell Biology, 2008. **10**(6): p. 635-7.
95. Wilkinson, K.A. and J.M. Henley, *Mechanisms, regulation and consequences of protein SUMOylation.* The Biochemical Journal, 2010. **428**(2): p. 133-145.
96. Hay, R.T., *SUMO: a history of modification.* Molecular Cell, 2005. **18**(1): p. 1-12.
97. Ulrich, H.D., *SUMO Modification: Wrestling with Protein Conformation.* Current Biology, 2005. **15**(7): p. R257-R259.
98. Everett, R.D., C. Boutell, and B.G. Hale, *Interplay between viruses and host sumoylation pathways.* Nature Reviews Microbiology, 2013. **11**(6): p. 400-411.

99. Yeh, E.T., *SUMOylation and De-SUMOylation: wrestling with life's processes.* The Journal of Biological Chemistry, 2009. **284**(13): p. 8223-7.
100. Sampson, D.A., M. Wang, and M.J. Matunis, *The Small Ubiquitin-like Modifier-1 (SUMO-1) Consensus Sequence Mediates Ubc9 Binding and Is Essential for SUMO-1 Modification.* The Journal of Biological Chemistry, 2001. **276**(24): p. 21664-21669.
101. Rodriguez, M.S., C. Dargemont, and R.T. Hay, *SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting.* The Journal of Biological Chemistry, 2001. **276**(16): p. 12654-9.
102. Kagey, M.H., T.A. Melhuish, and D. Wotton, *The Polycomb Protein Pc2 Is a SUMO E3.* Cell, 2003. **113**(1): p. 127-137.
103. Miyauchi, Y., et al., *Sumoylation of Mdm2 by Protein Inhibitor of Activated STAT (PIAS) and RanBP2 Enzymes.* The Journal of Biological Chemistry, 2002. **277**(51): p. 50131-50136.
104. Jang, M.-S., S.-W. Ryu, and E. Kim, *Modification of Daxx by small ubiquitin-related modifier-1.* Biochemical and Biophysical Research Communications, 2002. **295**(2): p. 495-500.
105. Le, N.-T., et al., *Reactive Oxygen Species, SUMOylation, and Endothelial Inflammation.* International Journal of Inflammation, 2012. **2012**: p. 13.
106. Jentsch, S., *The Ubiquitin-Conjugation System.* Annual Review of Genetics, 1992. **26**(1): p. 179-207.
107. Müller, S., et al., *Sumo, ubiquitin's mysterious cousin.* Nature Reviews Molecular Cell Biology, 2001. **2**(3): p. 202-213.
108. Lee, J.-S. and S.S. Thorgeirsson, *Genome-scale profiling of gene expression in hepatocellular carcinoma: Classification, survival prediction, and identification of therapeutic targets.* Gastroenterology, 2004. **127**(5, Supplement 1): p. S51-S55.
109. McDoniels-Silvers, A.L., et al., *Differential Gene Expression in Human Lung Adenocarcinomas and Squamous Cell Carcinomas.* Clinical Cancer Research, 2002. **8**(4): p. 1127-1138.
110. Mo, Y.-Y., et al., *A role for Ubc9 in tumorigenesis.* Oncogene, 2005. **24**(16): p. 2677-2683.
111. Wang, L. and S. Banerjee, *Differential PIAS3 expression in human malignancy.* Oncology Reports, 2004. **11**(6): p. 1319-1324.
112. Seeler, J.S., et al., *SUMO, the Three Rs and Cancer*, in *Acute Promyelocytic Leukemia*, P. Pandolfi and P. Vogt, Editors. 2007, Springer Berlin Heidelberg. p. 49-71.
113. Cheng, J., et al., *Role of Desumoylation in the Development of Prostate Cancer.* Neoplasia, 2006. **8**(8): p. 667-676.
114. Jacques, C., et al., *Two-Step Differential Expression Analysis Reveals a New Set of Genes Involved in Thyroid Oncocytic Tumors.* The Journal of Clinical Endocrinology & Metabolism, 2005. **90**(4): p. 2314-2320.
115. Steffan, J.S., et al., *SUMO Modification of Huntingtin and Huntington's Disease Pathology.* Science, 2004. **304**(5667): p. 100-104.
116. Phizicky, E.M. and S. Fields, *Protein-protein interactions: methods for detection and analysis.* Microbiological Reviews, 1995. **59**(1): p. 94-123.
117. Bonetta, L., *Protein-protein interactions: Interactome under construction.* Nature, 2010. **468**(7325): p. 851-854.
118. Vidal, M., Michael E. Cusick, and A.-L. Barabási, *Interactome Networks and Human Disease.* Cell, 2011. **144**(6): p. 986-998.

119. Milroy, L.-G., et al., *Modulators of Protein–Protein Interactions.* Chemical Reviews, 2014. **114**(9): p. 4695-4748.
120. Jaeger, S. and P. Aloy, *From protein interaction networks to novel therapeutic strategies.* IUBMB Life, 2012. **64**(6): p. 529-537.
121. Latchman, D.S., *Transcriptional Gene Regulation in Eukaryotes*, in *eLS*. 2011, John Wiley & Sons, Ltd.
122. Latchman, D.S., *Transcription factors: An overview.* The International Journal of Biochemistry & Cell Biology, 1997. **29**(12): p. 1305-1312.
123. Lee, T.I. and R.A. Young, *Transcription of Eukaryotic Protein-Coding Genes.* Annual Review of Genetics, 2000. **34**(1): p. 77-137.
124. Novina, C.D. and A.L. Roy, *Core promoters and transcriptional control.* Trends in Genetics, 1996. **12**(9): p. 351-355.
125. Geng, Y. and L.F. Johnson, *Lack of an initiator element is responsible for multiple transcriptional initiation sites of the TATA-less mouse thymidylate synthase promoter.* Molecular and Cellular Biology, 1993. **13**(8): p. 4894-4903.
126. Ince, T.A. and K.W. Scotto, *Differential utilization of multiple transcription start points accompanies the overexpression of the P-glycoprotein-encoding gene in Chinese hamster lung cells.* Gene, 1995. **156**(2): p. 287-290.
127. Lu, J., et al., *Start site selection by Sp1 in the TATA-less human Ha-ras promoter.* The Journal of Biological Chemistry, 1994. **269**(7): p. 5391-5402.
128. Ogbourne, S. and T.M. Antalis, *Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes.* Biochemical Journal, 1998. **331**(Pt 1): p. 1-14.
129. Hanna-Rose, W. and U. Hansen, *Active repression mechanisms of eukaryotic transcription repressors.* Trends in Genetics, 1996. **12**(6): p. 229-234.
130. Jongstra, J., et al., *A new lymphocyte-specific gene which encodes a putative Ca2+-binding protein is not expressed in transformed T lymphocyte lines.* The Journal of Immunology, 1988. **141**(11): p. 3999-4004.
131. Jongstra-Bilen, J., et al., *Human and mouse LSP1 genes code for highly conserved phosphoproteins.* The Journal of Immunology, 1990. **144**(3): p. 1104-10.
132. Pulford, K., et al., *Lymphocyte-specific protein 1: a specific marker of human leucocytes.* Immunology, 1999. **96**(2): p. 262-71.
133. Jongstra, J., et al., *The LSP1 gene is expressed in cultured normal and transformed mouse macrophages.* Molecular Immunology, 1994. **31**(15): p. 1125-31.
134. Li, Y., A. Guerrero, and T.H. Howard, *The actin-binding protein, lymphocyte-specific protein 1, is expressed in human leukocytes and human myeloid and lymphoid cell lines.* The Journal of Immunology, 1995. **155**(7): p. 3563-9.
135. Jongstra-Bilen, J., et al., *LSP1 modulates leukocyte populations in resting and inflamed peritoneum.* Blood, 2000. **96**(5): p. 1827-1835.
136. Liu, L., et al., *LSP1 is an endothelial gatekeeper of leukocyte transendothelial migration.* The Journal of Experimental Medicine, 2005. **201**(3): p. 409-18.
137. Kaur, J., *The Role of Endothelial Leukocyte Specific Protein-1 in the Inflammatory Response*, in *Department of Medical Science,* 2007, University of Calgary Calgary, Alberta. p. 189.
138. Klein, D.P., et al., *Lymphocyte-specific Ca2+-binding protein LSP1 is associated with the cytoplasmic face of the plasma membrane.* Molecular and Cellular Biology, 1989. **9**(7): p. 3043-8.

139. Klein, D.P., S. Galea, and J. Jongstra, *The lymphocyte-specific protein LSP1 is associated with the cytoskeleton and co-caps with membrane IgM.* The Journal of Immunology, 1990. **145**(9): p. 2967-73.
140. Jongstra-Bilen, J., et al., *The lymphocyte-specific protein LSP1 binds to F-actin and to the cytoskeleton through its COOH-terminal basic domain.* The Journal of Cell Biology, 1992. **118**(6): p. 1443-53.
141. Zhang, Q., Y. Li, and T.H. Howard, *Human lymphocyte-specific protein 1, the protein overexpressed in neutrophil actin dysfunction with 47-kDa and 89-kDa protein abnormalities (NAD 47/89), has multiple F-actin binding domains.* The Journal of Immunology, 2000. **165**(4): p. 2052-8.
142. Kadiyala, R.K., B.W. McIntyre, and A.M. Krensky, *Molecular cloning and characterization of WP34, a phosphorylated human lymphocyte differentiation and activation antigen.* European Journal of Immunology, 1990. **20**(11): p. 2417-23.
143. Huang, C.K., et al., *LSP1 is the major substrate for mitogen-activated protein kinase-activated protein kinase 2 in human neutrophils.* The Journal of Biological Chemistry, 1997. **272**(1): p. 17-9.
144. Omori, S.A., et al., *Differential interaction of nuclear factors with the leukocyte-specific pp52 promoter in B and T cells.* The Journal of Immunology, 1997. **159**(4): p. 1800-8.
145. Malone, C.S., et al., *Leukocyte-specific expression of the pp52 (LSP1) promoter is controlled by the cis-acting pp52 silencer and anti-silencer elements.* Gene, 2001. **268**(1–2): p. 9-16.
146. Hannigan, M., et al., *Leukocyte-specific gene 1 protein (LSP1) is involved in chemokine KC-activated cytoskeletal reorganization in murine neutrophils in vitro.* Journal of Leukocyte Biology, 2001. **69**(3): p. 497-504.
147. Wang, C., et al., *Modulation of Mac-1 (CD11b/CD18)-mediated adhesion by the leukocyte-specific protein 1 is key to its role in neutrophil polarization and chemotaxis.* The Journal of Immunology, 2002. **169**(1): p. 415-23.
148. Jongstra-Bilen, J., et al., *LSP1 modulates leukocyte populations in resting and inflamed peritoneum.* Blood, 2000. **96**(5): p. 1827-35.
149. Hannigan, M.O., et al., *Abnormal migration phenotype of mitogen-activated protein kinase-activated protein kinase 2-/- neutrophils in Zigmond chambers containing formyl-methionyl-leucyl-phenylalanine gradients.* The Journal of Immunology, 2001. **167**(7): p. 3953-61.
150. Zu, Y.L., et al., *p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or FMLP stimulation.* The Journal of Immunology, 1998. **160**(4): p. 1982-9.
151. Cara, D.C., et al., *Role of p38 mitogen-activated protein kinase in chemokine-induced emigration and chemotaxis in vivo.* The Journal of Immunology, 2001. **167**(11): p. 6552-8.
152. Carballo, E., et al., *Characterization and purification of a protein kinase C substrate in human B cells. Identification as lymphocyte-specific protein 1 (LSP1).* The Journal of Immunology, 1996. **156**(5): p. 1709-13.
153. Matsumoto, N., et al., *Protein kinase C phosphorylates p50 LSP1 and induces translocation of p50 LSP1 in T lymphocytes.* The Journal of Biochemistry, 1995. **117**(1): p. 222-9.

154. Coates, T.D., et al., *An inherited defect of neutrophil motility and microfilamentous cytoskeleton associated with abnormalities in 47-Kd and 89-Kd proteins.* Blood, 1991. **78**(5): p. 1338-46.
155. Howard, T., et al., *The 47-kD protein increased in neutrophil actin dysfunction with 47- and 89-kD protein abnormalities is lymphocyte-specific protein.* Blood, 1994. **83**(1): p. 231-41.
156. Howard, T.H., J. Hartwig, and C. Cunningham, *Lymphocyte-specific protein 1 expression in eukaryotic cells reproduces the morphologic and motile abnormality of NAD 47/89 neutrophils.* Blood, 1998. **91**(12): p. 4786-95.
157. Li, Y., et al., *LSP1 modulates the locomotion of monocyte-differentiated U937 cells.* Blood, 2000. **96**(3): p. 1100-5.
158. Miyoshi, E.K., et al., *Aberrant expression and localization of the cytoskeleton-binding pp52 (LSP1) protein in hairy cell leukemia.* Leukemia Research, 2001. **25**(1): p. 57-67.
159. Chen, M.-B., et al., *Association of a LSP1 gene rs3817198T>C polymorphism with breast cancer risk: evidence from 33,920 cases and 35,671 controls.* Molecular Biology Reports, 2011. **38**(7): p. 4687-4695.
160. Gorodnova, T.V., et al., *Distribution of FGFR2, TNRC9, MAP3K1, LSP1, and 8q24 alleles in genetically enriched breast cancer patients versus elderly tumor-free women.* Cancer Genetics and Cytogenetics. **199**(1): p. 69-72.
161. Koral, K., et al., *Leukocyte-Specific Protein 1: ANovel Regulator of Hepatocellular Proliferation and Migration Deleted in Human Hepatocellular Carcinoma.* Hepatology, 2015. **61**(2): p. 537-547.
162. Cao, M.Y., et al., *Inhibition of anti-IgM-induced translocation of protein kinase C beta I inhibits ERK2 activation and increases apoptosis.* The Journal of Biological Chemistry, 2001. **276**(27): p. 24506-10.
163. Harrison, R.E., B.A. Sikorski, and J. Jongstra, *Leukocyte-specific protein 1 targets the ERK/MAP kinase scaffold protein KSR and MEK1 and ERK2 to the actin cytoskeleton.* Journal of Cell Science, 2004. **117**(Pt 10): p. 2151-7.
164. Smith, A.L., et al., *Leukocyte-specific protein 1 interacts with DC-SIGN and mediates transport of HIV to the proteasome in dendritic cells.* The Journal of Experimental Medicine, 2007. **204**(2): p. 421-430.
165. Gringhuis, S.I., et al., *Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori.* Nature Immunology, 2009. **10**(10): p. 1081-1088.
166. Maxeiner, S., et al., *Crucial role for the LSP1–myosin1e bimolecular complex in the regulation of Fcγ receptor–driven phagocytosis.* Molecular Biology of the Cell, 2015. **26**(9): p. 1652-1664.
167. Gautreau, A., et al., *Ezrin, a plasma membrane-microfilament linker, signals cell survival through the phosphatidylinositol 3-kinase/Akt pathway.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(13): p. 7300-5.
168. Mangeat, P., C. Roy, and M. Martin, *ERM proteins in cell adhesion and membrane dynamics.* Trends in Cell Biology, 1999. **9**(5): p. 187-92.
169. Bretscher, A., K. Edwards, and R.G. Fehon, *ERM proteins and merlin: integrators at the cell cortex.* Nature Reviews Molecular Cell Biology, 2002. **3**(8): p. 586-99.

170. Gautreau, A., D. Louvard, and M. Arpin, *ERM proteins and NF2 tumor suppressor: the Yin and Yang of cortical actin organization and cell growth signaling.* Current Opinion in Cell Biology, 2002. **14**(1): p. 104-9.
171. Hughes, S.C. and R.G. Fehon, *Understanding ERM proteins--the awesome power of genetics finally brought to bear.* Current Opinion in Cell Biology, 2007. **19**(1): p. 51-6.
172. Gary, R. and A. Bretscher, *Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site.* Molecular Biology of the Cell, 1995. **6**(8): p. 1061-75.
173. Reczek, D. and A. Bretscher, *The carboxyl-terminal region of EBP50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule.* The Journal of Biological Chemistry, 1998. **273**(29): p. 18452-8.
174. Yonemura, S., et al., *Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2.* The Journal of Cell Biology, 1998. **140**(4): p. 885-95.
175. Fehon, R.G., A.I. McClatchey, and A. Bretscher, *Organizing the cell cortex: the role of ERM proteins.* Nature Reviews Molecular Cell Biology, 2010. **11**(4): p. 276-87.
176. Alcaide, P., S. Auerbach, and F.W. Luscinskas, *Neutrophil recruitment under shear flow: it's all about endothelial cell rings and gaps.* Microcirculation, 2009. **16**(1): p. 43-57.
177. Weinman, E.J., et al., *The association of NHERF adaptor proteins with g protein-coupled receptors and receptor tyrosine kinases.* Annual Review of Physiology, 2006. **68**: p. 491-505.
178. Barreiro, O., et al., *Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes.* The Journal of Cell Biology, 2002. **157**(7): p. 1233-45.
179. Lee, J.H., et al., *Roles of p-ERM and Rho-ROCK signaling in lymphocyte polarity and uropod formation.* The Journal of Cell Biology, 2004. **167**(2): p. 327-37.
180. Guo, X., et al., *ERM protein moesin is phosphorylated by advanced glycation end products and modulates endothelial permeability.* American Journal of Physiology - Heart and Circulatory Physiology, 2009. **297**(1): p. H238-H246.
181. Adyshev, D.M., et al., *Ezrin/radixin/moesin proteins differentially regulate endothelial hyperpermeability after thrombin.* American Journal of Physiology - Lung Cellular and Molecular Physiology, 2013. **305**(3): p. L240-L255.
182. Matsumoto, N., et al., *Lymphocyte isoforms of mouse p50 LSP1, which are phosphorylated in mitogen-activated T cells, are formed through alternative splicing and phosphorylation.* The Journal of Biochemistry, 1995. **118**(1): p. 237-43.
183. DeKroon, R.M., et al., *Analysis of protein posttranslational modifications using DIGE-based proteomics.* Methods in Molecular Biology, 2012. **854**: p. 129-43.
184. Du, J.X., et al., *SUMOylation regulates nuclear localization of Kruppel-like factor 5.* The Journal of Biological Chemistry, 2008. **283**(46): p. 31991-2002.
185. Chen, L., et al., *Sumoylation regulates nuclear localization and function of zinc finger transcription factor ZIC3.* Biochimica et Biophysica Acta, 2013. **1833**(12): p. 2725-33.
186. Carvalho-Tavares, J., et al., *A Role for Platelets and Endothelial Selectins in Tumor Necrosis Factor-α–Induced Leukocyte Recruitment in the Brain Microvasculature.* Circulation Research, 2000. **87**(12): p. 1141-1148.
187. Cooper, G.M. and R.E. Hausman, *Regulation of Transcription in Eukaryotes.*, in *The Cell: A Molecular Approach*. 2013, Sinauer Associates: Sunderland, MA. p. 832

188. Liang, C.-C., A.Y. Park, and J.-L. Guan, *In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro.* Nat. Protocols, 2007. **2**(2): p. 329-333.
189. Miah, S., et al., *BRK Targets Dok1 for Ubiquitin-Mediated Proteasomal Degradation to Promote Cell Proliferation and Migration.* PLoS ONE, 2014. **9**(2): p. e87684.
190. Salcedo, R., et al., *Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression.* Blood, 2000. **96**(1): p. 34-40.
191. Sivamurthy, N., et al., *Apolipoprotein J inhibits the migration and adhesion of endothelial cells.* Surgery, 2001. **130**(2): p. 204-209.
192. Şahin, M., E. Şahin, and S. Gümüşlü, *Effects of lycopene and apigenin on human umbilical vein endothelial cells in vitro under angiogenic stimulation.* Acta Histochemica, 2012. **114**(2): p. 94-100.
193. Shizukuda, Y., et al., *Vascular Endothelial Growth Factor–Induced Endothelial Cell Migration and Proliferation Depend on a Nitric Oxide–Mediated Decrease in Protein Kinase Cδ Activity.* Circulation Research, 1999. **85**(3): p. 247-256.
194. He, R., et al., *Inhibition of K562 leukemia angiogenesis and growth by expression of antisense vascular endothelial growth factor (VEGF) sequence.* Cancer Gene Ther, 2003. **10**(12): p. 879-886.
195. Shin, H.-S., et al., *Bacterial Lipoprotein TLR2 Agonists Broadly Modulate Endothelial Function and Coagulation Pathways In Vitro and In Vivo.* The Journal of Immunology, 2011. **186**(2): p. 1119-1130.
196. Chandran, S., et al., *Neural precursor cell-expressed developmentally down-regulated protein 4-2 (Nedd4-2) regulation by 14-3-3 protein binding at canonical serum and glucocorticoid kinase 1 (SGK1) phosphorylation sites.* The Journal of Biological Chemistry, 2011. **286**(43): p. 37830-40.
197. Ramakrishna, S., Y.H. Kim, and H. Kim, *Stability of zinc finger nuclease protein is enhanced by the proteasome inhibitor MG132.* PLOS ONE, 2013. **8**(1): p. e54282.
198. Cadete, V.J.J., et al., *Combined subthreshold dose inhibition of myosin light chain phosphorylation and MMP-2 activity provides cardioprotection from ischaemic/reperfusion injury in isolated rat heart.* British Journal of Pharmacology, 2013. **170**(2): p. 380-390.
199. Su, Y., et al., *Regulation of methylglyoxal-elicited leukocyte recruitment by endothelial SGK1/GSK3 signaling.* Biochim Biophys Acta, 2014. **1843**(11): p. 2481-91.
200. Li, Y., et al., *LSP1 modulates the locomotion of monocyte-differentiated U937 cells.* Bood, 2000. **96**(3): p. 1100-1105.
201. Risau, W., *Mechanisms of angiogenesis.* Nature, 1997. **386**(6626): p. 671-674.
202. Wang, S., et al., *Control of endothelial cell proliferation and migration by VEGF signaling to histone deacetylase 7.* Proceedings of the National Academy of Sciences, 2008. **105**(22): p. 7738-7743.
203. Osborn, L., et al., *Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes.* Cell, 1989. **59**(6): p. 1203-1211.
204. Rice, G.E., J. M. Munro, and M. P. Bevilacqua., *Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes. A CD11/CD18-independent adhesion mechanism.* The Journal of Experimental Medicine, 1990. **171**(4): p. 1369-1374.

205. Neish, A.S., et al., *Functional analysis of the human vascular cell adhesion molecule 1 promoter.* J Exp Med, 1992. **176**(6): p. 1583-93.
206. Barreiro, O., et al., *Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes.* J Cell Biol, 2002. **157**(7): p. 1233-45.
207. Sumagin, R., J.M. Kuebel, and I.H. Sarelius, *Leukocyte rolling and adhesion both contribute to regulation of microvascular permeability to albumin via ligation of ICAM-1.* American Journal of Physiology - Cell Physiology, 2011. **301**(4): p. C804-C813.
208. Wu, Y., et al., *MAPKAPK2-mediated LSP1 phosphorylation and FMLP-induced neutrophil polarization.* Biochemical and Biophysical Research Communications, 2007. **358**(1): p. 170-5.
209. Phillipson, M., et al., *Endothelial Domes Encapsulate Adherent Neutrophils and Minimize Increases in Vascular Permeability in Paracellular and Transcellular Emigration.* PLoS ONE, 2008. **3**(2): p. e1649.
210. Wang, Q. and C.M. Doerschuk, *The p38 Mitogen-Activated Protein Kinase Mediates Cytoskeletal Remodeling in Pulmonary Microvascular Endothelial Cells Upon Intracellular Adhesion Molecule-1 Ligation.* The Journal of Immunology, 2001. **166**(11): p. 6877-6884.
211. Maspero, E., et al., *Structure of the HECT:ubiquitin complex and its role in ubiquitin chain elongation.* Embo Reports 2011. **12**(4): p. 342-349.
212. Rothermel, T.A., B. Engelhardt, and N. Sheibani, *Polyoma virus middle-T-transformed PECAM-1 deficient mouse brain endothelial cells proliferate rapidly in culture and form hemangiomas in mice.* Journal of Cellular Physiology, 2005. **202**(1): p. 230-239.
213. GRATZINGER, D., et al., *Platelet endothelial cell adhesion molecule-1 modulates endothelial cell motility through the small G-protein Rho.* The FASEB Journal, 2003. **17**(11): p. 1458-1469.
214. Tsuneki, M. and J.A. Madri, *CD44 Regulation of Endothelial Cell Proliferation and Apoptosis via Modulation of CD31 and VE-cadherin Expression.* Journal of Biological Chemistry, 2014. **289**(9): p. 5357-5370.
215. Jongstra-Bilen, J., et al., *LSP1 regulates anti-IgM induced apoptosis in WEHI-231 cells and normal immature B-cells.* Molecular Immunology, 1999. **36**(6): p. 349-359.
216. Lei, X., et al., *Different microvascular permeability responses elicited by the CXC chemokines MIP-2 and KC during leukocyte recruitment: role of LSP1.* Biochemical and Biophysical Research Communications, 2012. **423**(3): p. 484-9.
217. Sheibani, N. and W.A. Frazier, *Down-Regulation of Platelet Endothelial Cell Adhesion Molecule-1 Results in Thrombospondin-1 Expression and Concerted Regulation of Endothelial Cell Phenotype.* Molecular Biology of the Cell, 1998. **9**(4): p. 701-713.
218. Sheibani, N., P.J. Newman, and W.A. Frazier, *Thrombospondin-1, a natural inhibitor of angiogenesis, regulates platelet-endothelial cell adhesion molecule-1 expression and endothelial cell morphogenesis.* Molecular Biology of the Cell, 1997. **8**(7): p. 1329-1341.
219. Solowiej, A., et al., *Lack of Platelet Endothelial Cell Adhesion Molecule-1 Attenuates Foreign Body Inflammation because of Decreased Angiogenesis.* The American Journal of Pathology, 2002. **162**(3): p. 953-962.
220. Biswas, P., et al., *PECAM-1 promotes β-catenin accumulation and stimulates endothelial cell proliferation.* Biochemical and Biophysical Research Communications, 2003. **303**(1): p. 212-218.

221. Graesser, D., et al., *Altered vascular permeability and early onset of experimental autoimmune encephalomyelitis in PECAM-1–deficient mice.* The Journal of Clinical Investigation, 2002. **109**(3): p. 383-392.
222. Kanki, Y., et al., *Epigenetically coordinated GATA2 binding is necessary for endothelium-specific endomucin expression.* EMBO J, 2011. **30**(13): p. 2582-95.
223. van Buul, J.D., et al., *Inside-Out Regulation of ICAM-1 Dynamics in TNF-α-Activated Endothelium.* PLOS ONE, 2010. **5**(6): p. e11336.
224. Williams, M.R. and F.W. Luscinskas, *Leukocyte rolling and adhesion via ICAM-1 signals to endothelial permeability. Focus on "Leukocyte rolling and adhesion both contribute to regulation of microvascular permeability to albumin via ligation of ICAM-1".* American Journal of Physiology - Cell Physiology, 2011. **301**(4): p. C777-C779.
225. Ubersax, J.A. and J.E. Ferrell Jr, *Mechanisms of specificity in protein phosphorylation.* Nature Reviews Molecular Cell Biology, 2007. **8**(7): p. 530-541.
226. Nishi, H., K. Hashimoto, and Anna R. Panchenko, *Phosphorylation in Protein-Protein Binding: Effect on Stability and Function.* Structure, 2011. **19**(12): p. 1807-1815.
227. Johnson, Louise N., *The regulation of protein phosphorylation.* Biochemical Society Transactions, 2009. **37**(4): p. 627-641.
228. Olsen, J.V., et al., *Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks.* Cell, 2006. **127**(3): p. 635-648.
229. Olsen, J.V., et al., *Quantitative Phosphoproteomics Reveals Widespread Full Phosphorylation Site Occupancy During Mitosis.* Science Signaling, 2010. **3**(104): p. ra3-ra3.
230. Jenal, U. and M.Y. Galperin, *Single domain response regulators: molecular switches with emerging roles in cell organization and dynamics.* Current Opinion in Microbiology, 2009. **12**(2): p. 152-160.
231. Lin, K., P.K. Hwang, and R.J. Fletterick, *Distinct phosphorylation signals converge at the catalytic center in glycogen phosphorylases.* Structure, 1997. **5**(11): p. 1511-1523.
232. Lima, C.D. and D. Reverter, *Structure of the human SENP7 catalytic domain and poly-SUMO deconjugation activities for SENP6 and SENP7.* The Journal of Biological Chemistry, 2008. **283**(46): p. 32045-55.
233. Sharma, P., et al., *Senp1 is essential for desumoylating Sumo1-modified proteins but dispensable for Sumo2 and Sumo3 deconjugation in the mouse embryo.* Cell Reports, 2013. **3**(5): p. 1640-50.
234. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-141.
235. Matic, I., et al., *Site-specific identification of SUMO-2 targets in cells reveals an inverted SUMOylation motif and a hydrophobic cluster SUMOylation motif.* Molecular Cell, 2010. **39**(4): p. 641-52.
236. Watanabe, M. and K. Itoh, *Characterization of a novel posttranslational modification in neuronal nitric oxide synthase by small ubiquitin-related modifier-1.* Biochimica et Biophysica Acta, 2011. **1814**(7): p. 900-7.
237. Palczewska, M., et al., *Sumoylation regulates nuclear localization of repressor DREAM.* Biochimica et Biophysica Acta, 2011. **1813**(5): p. 1050-8.
238. Xu, K., et al., *Modification of nonstructural protein 1 of influenza A virus by SUMO1.* Journal of Virology, 2011. **85**(2): p. 1086-98.

239. Miles, W.O., et al., *Medea SUMOylation restricts the signaling range of the Dpp morphogen in the Drosophila embryo.* Genes & Development, 2008. **22**(18): p. 2578-90.
240. Klenk, C., et al., *SUMO-1 controls the protein stability and the biological function of phosducin.* The Journal of Biological Chemistry, 2006. **281**(13): p. 8357-64.
241. Lin, X., et al., *SUMO-1/Ubc9 Promotes Nuclear Accumulation and Metabolic Stability of Tumor Suppressor Smad4.* The Journal of Biological Chemistry, 2003. **278**(33): p. 31043-31048.
242. Bies, J., J. Markus, and L. Wolff, *Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity.* The Journal of Biological Chemistry, 2002. **277**(11): p. 8999-9009.
243. Berkholz, J., L. Michalick, and B. Munz, *The E3 SUMO ligase Nse2 regulates sumoylation and nuclear-to-cytoplasmic translocation of skNAC-Smyd1 in myogenesis.* Journal of Cell Science, 2014. **127**(Pt 17): p. 3794-804.
244. Giagulli, C., et al., *RhoA and ζ PKC Control Distinct Modalities of LFA-1 Activation by Chemokines: Critical Role of LFA-1 Affinity Triggering in Lymphocyte In Vivo Homing.* Immunity, 2004. **20**(1): p. 25-35.
245. Grethe, S., et al., *p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-xL.* Experimental Cell Research, 2004. **298**(2): p. 632-642.
246. Li, G., et al., *Tumor Necrosis Factor-α Induces Insulin Resistance in Endothelial Cells via a p38 Mitogen-Activated Protein Kinase-Dependent Pathway.* Endocrinology, 2007. **148**(7): p. 3356-3363.
247. Cinamon, G., et al., *Chemoattractant Signals and β2 Integrin Occupancy at Apical Endothelial Contacts Combine with Shear Stress Signals to Promote Transendothelial Neutrophil Migration.* The Journal of Immunology, 2004. **173**(12): p. 7282-7291.
248. Schmidt, E.P., et al., *On, Around, and Through: Neutrophil-Endothelial Interactions in Innate Immunity.* Physiology (Bethesda, Md.), 2011. **26**(5): p. 334-347.
249. DiStasi, M.R. and K. Ley, *Opening the flood-gates: how neutrophil-endothelial interactions regulate permeability.* Trends in Immunology, 2009. **30**(11): p. 547-556.
250. Dull, R.O. and J.G.N. Garcia, *Leukocyte-Induced Microvascular Permeability: How Contractile Tweaks Lead to Leaks.* Circulation Research, 2002. **90**(11): p. 1143-1144.
251. Mei, H., et al., *Regulation of Endothelial Cell Barrier Function by Antibody-driven Affinity Modulation of PECAM-1.* The Journal of Biological Chemistry, 2014.
252. Turegun, M., et al., *Blockade of platelet endothelial cell adhesion molecule-1 (PECAM-1) protects against ischemia-reperfusion injury in muscle flaps at microcirculatory level.* Plastic and Reconstructive Surgery, 1999. **104**(4): p. 1033-40.
253. Privratsky, J.R., et al., *Relative contribution of PECAM-1 adhesion and signaling to the maintenance of vascular integrity.* Journal of Cell Science, 2011. **124**(Pt 9): p. 1477-85.
254. Vandenbroucke, E., et al., *Regulation of Endothelial Junctional Permeability.* Annals of the New York Academy of Sciences, 2008. **1123**(1): p. 134-145.
255. Meng, W., et al., *Structure of Mitogen-activated Protein Kinase-activated Protein (MAPKAP) Kinase 2 Suggests a Bifunctional Switch That Couples Kinase Activation with Nuclear Export.* The Journal of Biological Chemistry, 2002. **277**(40): p. 37401-37405.

256. Wood, L.D., et al., *Small ubiquitin-like modifier conjugation regulates nuclear export of TEL, a putative tumor suppressor.* Proceedings of the National Academy of Sciences, 2003. **100**(6): p. 3257-3262.
257. Santiago, A., et al., *p53 SUMOylation promotes its nuclear export by facilitating its release from the nuclear export receptor CRM1.* Molecular Biology of the Cell, 2013. **24**(17): p. 2739-52.
258. Hossain, M., et al., *Endothelial LSP1 Modulates Extravascular Neutrophil Chemotaxis by Regulating Nonhematopoietic Vascular PECAM-1 Expression.* The Journal of Immunology, 2015. **195**(5): p. 2408-16.
259. Goto, H., et al., *Gene therapy utilizing the Cre/loxP system selectively suppresses tumor growth of disseminated carcinoembryonic antigen-producing cancer cells.* International Journal of Cancer, 2001. **94**(3): p. 414-419.
260. Kijima, T., et al., *Application of the Cre Recombinase/loxP System Further Enhances Antitumor Effects in Cell Type-specific Gene Therapy against Carcinoembryonic Antigen-producing Cancer.* Cancer Research, 1999. **59**(19): p. 4906-4911.

APPENDIX 1 (ACADEMIC ACHIEVEMENTS)

RESEARCH PUBLICATIONS

Full length research papers

Manuscripts based on this thesis

1. **Mokarram Hossain**, Syed M. Qadri and Lixin Liu. ICAM-1 cross-linking-mediated LSP1-moesin interaction enhances vascular permeability. **Manuscript is ready to be submitted.**
2. **Mokarram Hossain**, Jiannan Huang, Francisco S Cayabyab and Lixin Liu. SUMOylation protects endothelial LSP1 from ubiquitination-mediated proteasomal degradation and helps its nuclear export. *Manuscript in revision.* **Journal of Biological Chemistry.**
3. **Mokarram Hossain***, Syed M. Qadri*, Najia Xu, Yang Su, Francisco S. Cayabyab, Bryan Heit and Lixin Liu. Endothelial LSP1 modulates extravascular neutrophil chemotaxis by regulating non-hematopoietic vascular PECAM-1 expression. **The Journal of Immunology**, 2015, 195:2408-2416. *Equal contribution.
4. **Mokarram Hossain**, Syed M. Qadri, Yang Su and Lixin Liu. ICAM-1-mediated leukocyte adhesion is critical for the activation of endothelial LSP1. **American Journal of Physiology-Cell Physiology**, 2013, 304: C895-C904.

Manuscripts not related to this thesis

1. **Mokarram Hossain**, Entesar Omran, Najia Xu and Lixin Liu. The specific mitogen- and stress-activated protein kinase MSK1 inhibitor SB-747651A modulates chemokine-induced neutrophil recruitment. **Manuscript is ready to be submitted.**
2. Le Nguyen Phuong Khanh, Shankaramurthy Channabasappa, **Mokarram Hossain**, Lixin Liu, and Baljit Singh. Leukocyte-specific protein 1 (LSP1) regulates neutrophil recruitment in acute lung inflammation. *Manuscript in press.* **American Journal of Physiology - Lung Cellular and Molecular Physiology.**
3. Yang Su*, Syed M. Qadri*, **Mokarram Hossain**, Lingyun Wu and Lixin Liu. Uncoupling of eNOS contributes to redox-sensitive leukocyte recruitment and microvascular leakage elicited by methylglyoxal. **Biochemical Pharmacology**, 2013, 86: 1762-1774. *Equal contribution.
4. Najia Xu, **Mokarram Hossain**, Lixin Liu. Pharmacological inhibition of p38 mitogen-activated protein kinases affects KC/CXCL1-induced intraluminal crawling, transendothelial migration, and chemotaxis of neutrophils *in vivo*. **Mediators of Inflammation**. Vol. 2013, Article ID 290565, doi:10.1155/2013/290565.
5. **Mokarram Hossain**, Syed M. Qadri and Lixin Liu. Inhibition of nitric oxide synthesis enhances leukocyte rolling and adhesion in human microvasculature. **Journal of Inflammation**, 2012, 9: 28-35.
6. Xi Lei, **Mokarram Hossain**, Syed M. Qadri and Lixin Liu. Different microvascular permeability responses elicited by the CXC chemokines MIP-2 and KC during leukocyte recruitment: role of LSP1. **Biochemical and Biophysical Research Communications,** 2012, 423: 484-489.

Abstracts

1. Syed M. Qadri, **Mokarram Hossain**, Najia Xu, Lixin Liu. Non-hematopoietic endothelial LSP1 orchestrates CXCL2-sensitive motile neutrophil functions *in vivo*. Paper presented at: The 15th International Congress of Immunology; 2013 Aug 22-27; Milan, Italy.
2. Syed M. Qadri, **Mokarram Hossain**, Najia Xu, Yang Su, Lixin Liu. Endothelial LSP1 regulates vascular CD31/PECAM-1 expression to foster alpha6beta1 integrin-dependent neutrophil chemotaxis *in vivo* [abstract]. Paper presented at: The 56th Annual Conference of the Canadian Society for Molecular Biosciences; 2013 Jun 3-7; Niagara, ON, Canada.
3. **Mokarram Hossain**, Jiannan Huang, Francisco S. Cayabyab, Lixin Liu. Sumoylation is crucial for the stability of Leukocyte-specific protein 1. Abstract published in The Journal of Immunology, 2013, 190, 58.13.Paper presented at: The 100th AAI Annual Meeting "IMMUNOLOGY 2013™", May 201, Honolulu, Hawaii, USA.
4. Syed M. Qadri, Najia Xu, **Mokarram Hossain**, Lixin Liu. Endothelial-expressed leukocyte-specific protein 1 regulates intravascular and extravascular directionality of chemotaxing neutrophils. Abstract published in The Journal of Immunology, 2013, 190, 58.1. Paper presented at: The 100th AAI Annual Meeting "IMMUNOLOGY 2013™", May 201, Honolulu, Hawaii, USA.
5. Lixin Liu, **Mokarram Hossain**, Syed M. Qadri. Pharmacological inhibition of p38 mitogen-activated protein kinases affects KC/CXCL1-induced intraluminal crawling, transendothelial migration, and chemotaxis of neutrophils *in vivo*. The FASEB Journal 2013; 27:646.3. Paper presented at: The Experimental Biology 2013; 2013 Apr 20-24; Boston, MA, USA.
6. **Mokarram Hossain,** Yang Su, Syed M. Qadri, Lixin Liu. ICAM-1-mediated leukocyte adhesion is critical for the activation of endothelial LSP1. In the Annual Meeting of the American Society for Cell Biology, December 15-19, 2012, San Francisco, USA.

ACADEMIC AWARDS AND HONOR

- Arthur Smyth memorial Scholarship provided by the college of Medicine, University of Saskatchewan for the period of September, 2015 to August, 2016.
- Graduate Teaching Fellowship (GTF) provided by the college of Graduate Studies and Research, University of Saskatchewan for the period of September, 2014 to August, 2015.
- Graduate Teaching Fellowship (GTF) provided by the college of Graduate Studies and Research, University of Saskatchewan for the period of September, 2013 to August, 2014.
- Dr. Jaques Memorial Scholarship for the period of September, 2012 to August, 2013.
- Department of Pharmacology Devolved Scholarship for the period of September, 2012 to August, 2013.
- Department of Pharmacology Devolved Scholarship for the period of September, 2011 to August, 2012.
- Achieved highest grade (92%) in Graduate Pharmacology (PCOL850) in 2010-2011 academic year.